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**Investigating the CEACAM1 adhesive properties of Moraxella catarrhalis**

*Identifying a novel CEACAM1 adhesion protein found in Mx13L*

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# INVESTIGATING THE CEACAM1 ADHESIVE PROPERTIES OF MORAXELLA CATARRHALIS

*IDENTIFYING A NOVEL CEACAM1 ADHESION PROTEIN FOUND IN MX13L*

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# 1 ABSTRACT

*Moraxella catarrhalis* is an upper respiratory pathogen often implicated as the cause otitis media infections, as well as exacerbating chronic obstructive pulmonary disease. One of the human receptors identified for *Moraxella catarrhalis* is Carcinoembryonic antigen-related cell adhesion molecule 1 (CC1), normally the surface adhesins UspA1 and UspA2V use the CC1 specific binding sequence within the previously identified rD-7region of UspA1 to interact with CC1. ATCC25240 is a strain of *Moraxella catarrhalis* which lacks the rD-7 region within its UspA1. This study revealed that a variant of ATCC25240 dubbed MX13L, which also lacks the rD-7 regions binds to CC1. The unknown adhesin present in MX13L interacted with CC1 in a heat modifiable manner. Using a combination of bioinformatics and the ez tn5 transposon to generate a transposon library of MX13L in conjunction with immune overlay assays, we identified that WP_003661028 (BOMP 8) as a putative candidate protein for the novel adhesin.

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# 3 AUTHOR'S DECLARATION

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's *Regulations and Code of Practice for Research Degree Programmes* and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: ..... DATE:.....

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# 5 ABBREVIATIONS

| | |
|---|---|
| **Amp** | Ampicillin |
| **AP** | Alkaline Phosphate |
| **APS** | Ammonium Persulfate |
| **BamA/TamA** | Outer Membrane Protein Assembly Factor Bama/ Translocation and Assembly Module Subunit |
| **BCA** | Bicinchoninic Acid Assay |
| **BCIP** | 5-Bromo-4-Chloro-3-Indolyl-Phosphate |
| **BHI** | Brain Heart Infusion |
| **BOMP** | Beta Barrel Outer Membrane Protein |
| **BRO-1 or BRO-2** | Class A Beta-Lactamase found in *M*. *catarrhalis* Type 1 Or 2 |
| **BSA** | Bovine Serum Albumin |

| | |
|---|---|
| **CC1** | Carcinoembryonic Antigen-Related Cell Adhesion Molecule 1 |
| **CEA** | Carcinoembryonic Antigen |
| **CEACAM** | Carcinoembryonic Antigen-Related Cell Adhesion Molecule |
| **CHO** | Chinese Hamster Ovary |
| **COPD** | Chronic Obstructive Pulmonary Disease |
| **CV** | Column Volumes |
| **DAPI** | 4′,6-Diamidino-2-Phenylindole |
| **DMSO** | Dimethyl Sulfoxide |
| **DNA** | Deoxyribonucleic Acid |
| **DPBS** | Dulbecco's Phosphate-Buffered Saline |
| **ECM** | Extracellular Matrix |
| **EDTA** | Ethylenediaminetetraacetic Acid |
| **FBS** | Fetal Bovine Serum |

| | |
|---|---|
| **Fc** | Fragment Crystallizable Region |
| **HBHI** | Brain Heart Infusion Broth Supplemented with Horse Blood |
| **IL2** | Interleukin-2 |
| **IPTG** | Isopropyl B- D-1-Thiogalactopyranoside |
| **ITSG** | Insulin-Transferrin-Selenium |
| **Kan** | Kanamycin |
| **Kdo** | 3-Deoxy-D-Manno-Octulosonic Acid |
| **KO** | Knock-Outs |
| **LB** | Lysogeny Broth |
| **LOS** | Lipid Oligosaccharides |
| **lpxA** | Acyl-[Acyl-Carrier-Protein]--UDP-N-Acetylglucosamine O-Acyltransferase |
| **McaP** | *Moraxella catarrhalis* Adherence Protein |

| | |
|---|---|
| **Mha** | M. *catarrhalis* Filamentous Hag (Fha)-Like Proteins |
| **MIC** | Minimum Inhibitory Concentration |
| **MID/Hag** | M. *catarrhalis* Immunoglobulin D (Igd) Binding Protein/Hemagglutein |
| **MOI** | Multiplicity of Infection |
| **MWCO** | Molecular Weight-Cutoff |
| **NBT** | Nitro-Blue Tetrazolium |
| **NCIB** | National Center For Biotechnology Information |
| **OD** | Optical Density |
| **OM** | Outer Membrane |
| **OMP** | Outer Membrane Protein |
| **OPA** | Opacity-Associated Proteins |
| **PBST** | Phosphate-Buffered Saline with Tween |

| | |
|---|---|
| **PCR** | Polymerase Chain Reaction |
| **Pen** | Penicillin |
| **PFA** | Paraformaldehyde |
| **PSG** | Pregnancy Specific Glycoproteins |
| **RPM** | Revolutions Per Minute |
| **RT** | Room Temperature |
| **SDS-PAGE** | Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis |
| **ShlB/FhaC/HecB** | Haemolysin Secretion/Activation Protein |
| **SOC** | Super Optimal Broth with Catabolite Repression |
| **TAA** | Trimeric Autotranspoters |
| **TBE** | Tris/Borate/EDTA |
| **TEMED** | Tetramethylethylenediamine |
| **TFP** | Type IV Pili |

| | |
|---|---|
| **TonB** | Outer Membrane Proteins that Bind and Transport Ferric Chelates |
| **TonB-tbp-lbp** | The TonB-Dependent Receptor Family |
| **Tris** | Trisaminomethane |
| **UspA** | Ubiquitous Surface Protein A |
| **X-Gal** | 5-Bromo-4-Chloro-3-Indolyl B-D-Galactopyranoside |
| **YadA** | Yersinia Adhesin A |
| **Zeo** | Zeocin |

# 1 INTRODUCTION

## 1.1 Background information

*Moraxella catarrhalis* is a gram-negative diplococcal bacterium of the order *Pseudomonadales*. It has been predominantly involved in upper respiratory tract infections such as otitis media but in recent years it has been implicated in the exacerbation of COPD. One of the major host cell receptors of *Moraxella catarrhalis* is human Carcinoembryonic Antigen-related Cell Adhesion Molecule 1 (CC1). Although Ubiquitous surface protein (Usp)A1 as well as UspA2V, identified as the main bacterial proteins which interact with CC1 are well understood, recent experiments have indicated that there may be another yet unknown CC1 binding protein. This project aims to identify the novel CC1 ligand.

## 1.2 Taxonomy

*Moraxella catarrhalis* was first described in 1896 by R.J. Pfeiffer, who named it *Micrococcus catarrhalis* (Murphy, 1998) (Catlin, 1990). Since the discovery this bacterium has been reclassified numerous times. First in 1963 when Burges nitrate reduction reactions showed that *Micrococcus catarrhalis* could be split into to two distinct species *Neisseria cinerea* and *N*. *catarrhalis* (Verduin et al., 2002) (Berger, 1963). Then in the 1970s when DNA hybridisation studies showed little homology with 'true' *Neisseria* species it was renamed *Branhamella catarrhalis* (Murphy, 1998) (Catlin, 1970)**.** In 1984 *B*. *catarrhalis* was reassigned to the genus *Moraxella* although many prefer the old name *Branhamella* and argued that the genus *Moraxella* should be split into two sub-genera (Bovre, 1979). The advent of 16S ribosomal DNA

sequencing proved that *Moraxella catarrhalis* was closely related to *M. lacunata* and more closely related to *Acinetobacter* than to *Neisseria* thus *Moraxella catarrhalis* is now the widely accepted name (Pettersson et al., 1998) (figure 1).

Along with determining that *Moraxella catarrhalis* is in fact a *Moraxella* species, 16s ribosomal data and restriction length polymorphism analysis has determined that there are 2 distinct evolutionary lineages of *Moraxella catarrhalis* (Bootsma et al., 2000). The seroresistant line contains 16S ribotype 1 strains, whilst the serosensitive strains have 16S ribotype 2 or 3 (Earl et al., 2016) (de Vries et al., 2013) (Bootsma et al., 2000). Of the two lineages the seroresistant strains have a been isolated from infected individuals, suggesting a more pathogenic nature, they display both efficient adherence to epithelial cells as well as complement resistance (Verhaegh et al., 2008).

Compared to the seroresistant strains the serosensitive strains display reduced adherence to respiratory epithelial cells as well as sensitivity to complement mediated killing. Although the seroresistant lineage is more pathogenic than the serosensitive lineages, whole genome analysis of clinically relevant seroresistant strains with varied disease profiles revealed limited genetic diversity between seroresistant strains (Wirth et al., 2007). In comparison the serosensitive lineage is much more genetically diverse and the genetic drift between strains appears to be mainly caused by point mutations, this suggests that the serosensitive lineages is the older population (Earl et al., 2016).

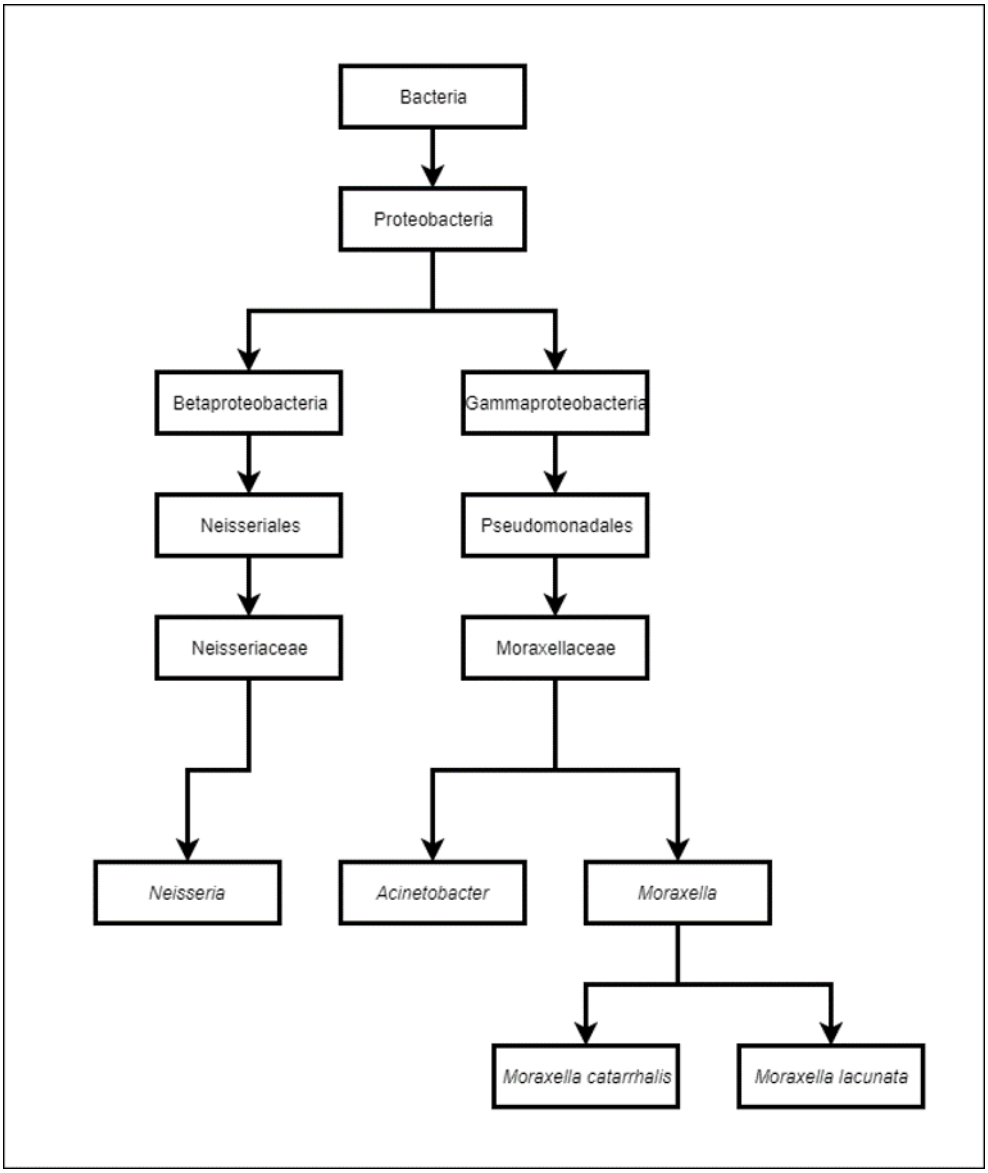



Figure 1 Taxonomy of *Moraxella catarrhalis*.

This image demonstrates the genus level relation between *Moraxella catarrhalis* and *M. lacunata*, and the familial relation between *Moraxella catarrhalis* and *Acinetobacter* species. The distant association of *Moraxella catarrhalis* and *Neisseria* species is also evident, as the species diverge at class level. These relationships can be confirmed by 16S ribosomal sequencing.

## 1.3 Epidemiology

Following its discovery this gram-negative diplococcal bacterium was regarded as a harmless commensal of the upper respiratory tract (Ariza-Prota et al., 2016). In the last few decades it has emerged as a significant pathogen of the upper and lower respiratory systems both in healthy and immunocompromised individuals (Verduin et al., 2002). The carriage rates of *Moraxella catarrhalis* are highest in children; 77% become colonised in the first five years, although only a small portion (approximately 9%) of these isolates are of clinical significance (Verduin et al., 2002). In contrast to the high carriage rates in children, *Moraxella catarrhalis* only colonises a small portion of adults (1-3%) but around 30% of these cases become clinically relevant (Vaneechoutte et al., 1990).

### 1.3.1 Diseases in children

In children this bacterium is one of the most prevalent causes of otitis media, along with *Streptococcus pneumoniae* and *Haemophilus influenzae*. Although estimates put *Moraxella catarrhalis* as the sole cause of 15-20% of acute otitis media cases, PCR detected in up to 47% of bacterial cultures obtained from middle ear fluid (Murphy and Parameswaran, 2009) (Sillanpää et al., 2016). Due to the age groups at most risk, and the recurrent nature of otitis media infections, it is a significant cause of morbidity and burden on health services, estimated to cost upwards of £200 million each year to treat (Williamson et al., 2009) (Francis et al., 2018).

### 1.3.2 Diseases in adults

Infections in adults tend towards those with already compromised immune systems, causing sinusitis in the upper respiratory tract and exacerbating chronic obstructive pulmonary disease (COPD) in the lower (Ramadan, Ibrahim, Shaheen and Ali, 2017). COPD is the fourth most common cause of death worldwide, and prevalence in the UK has increased by 27% in the last decade (WHO, 2016) (British Lung Foundation, 2019). Recent research has shown that *Moraxella catarrhalis* can be isolated more often from sputum samples than either *Streptococcus pneumoniae* or *Haemophilus influenzae*, colonising approximately 32% of patients with COPD (Parameswaran et al., 2009) (Leung et al., 2017) (Perez and Murphy, 2017). Although not all colonisation lead to exacerbation, about 10% of all COPD exacerbation is caused by *M. catarrhalis*. There are 1.2 million people living with COPD in the UK and exasperation caused by *Moraxella catarrhalis* costs the NHS approximately £190 million to treat per annum (British Lung Foundation, 2019).

### 1.3.3 Septicaemia and bacteraemia

Patients who are hospitalised for prolonged periods, along with those in intensive care units and the very young or elderly, often have a poor prognosis when suffering from a *Moraxella catarrhalis* infection (Constantinescu, 2019). Cases of bacteraemia, meningitis and sepsis has been reported within the at-risk groups. These patients have high mortality rates of about 21% (Ioannidis et al., 1995). When *Moraxella catarrhalis* causes bacteraemia, an associated respiratory infection is usually found. Severe bacteraemia has even been linked with the development of endocarditis (Ioannidis et

al., 1995). Although *Moraxella catarrhalis* survives in the bloodstream better than *Neisseria meningitidis,* cases of bacteraemia have been infrequently reported. This may be due to misdiagnosis as *Moraxella catarrhalis* is both very similar to *Neisseria* species and has only recently been identified as a significant pathogen.

## 1.4 Antibiotic resistance

Prior to its designation as a significant pathogen of the respiratory tract *Moraxella catarrhalis* was regarded as a harmless commensal which was easily treated via penicillin (Lacy, Berk and Berk, 2017). In the last 40 years this opinion has been revised. In 1976 a Swedish sputum isolate of *Moraxella catarrhalis* was reported to be resistant to penicillin and ampicillin (Malmvall, Brorsson and Johnsson, 1977). Further tests revealed it to be a novel beta-lactamase. The appearance of this novel resistance gene occurred practically simultaneously around the world and today over 95% of isolates produce one of 2 beta-lactamases; BRO-1 or BRO-2 (Shi et al., 2018) (Khan et al., 2009). Although these proteins are functionally identical with both genes binding the same substrate and only differing in sequence by a single amino acid residue (Glycine for Aspartate), BRO-1 confers a higher MIC than BRO-2 (Bootsma et al., 1996) (Uniprot, 2019). This difference in level of resistance is probably due to differences in the promoter regions resulting in a 3-fold increase in BRO-1 production (Schmitz et al., 2002).

Along with beta lactam resistance, *Moraxella catarrhalis* is also resistant to some second generation cephalosporins. Approximately 80% of isolates obtained from the

UK demonstrate resistance to cefaclor and 5% are resistant to cefuroxime (Khan et al., 2009).

## 1.5 Vaccine development

*Moraxella catarrhalis* is a clinically relevant pathogen which causes significant morbidity and mortality, as well as burdening health care systems worldwide (Perez and Murphy, 2019). Due to the widespread resistance to beta lactams within the *Moraxella catarrhalis* population as well as rising resistance to second generation antibiotics, it has become evident that a preventative vaccine is necessary.

### 1.5.1 Challenges

When designing a vaccine, it is important to consider the following criteria for antigen selection (Thibau et al., 2019) (Ren and Pichichero, 2015) (Raynes et al., 2018):

- *Is the epitope expressed on the surface of the bacteria?*
- *Is the epitope conserved among different strains?*
- *Is the antigen expressed in vivo at sites of pathogenesis?*
- *Is the antigen immunogenic?*
- *Does the antigen induce a protective immune response?*

With regards to *Moraxella catarrhalis* there are a number of limitations to the development of an effective vaccine (Ren and Pichichero, 2015). In silico and in vitro studies of *Moraxella catarrhalis* have identified many adhesins (see 1.6) which satisfy the first three criteria for antigen selection (Grandi, 2010). Although these antigens have been shown to be immunogenic, very few of the antibodies generated have been assessed for functionality (Perez and Murphy, 2017). Another factor is that there is no set of standard test parameters established by which to assess the efficacy of potential

antibodies. Another factor to consider is that many of the identified surface antigens such as UspA and MID/Hag are subject to phase variation. Another limitation to the development of a vaccine is the lack of animal models (Perez and Murphy, 2017). *Moraxella catarrhalis* is a human restricted pathogen which means that it is difficult to study the effects of a potential vaccine candidate *in vivo*.

The main animal model used to study *Moraxella catarrhalis* infections *in vivo* is via inoculating mice (Ruckdeschel, Brauer, Johnson and Murphy, 2009). Unfortunately, it is not possible to colonise mice with *Moraxella catarrhalis* and naive mice will usually clear a *Moraxella catarrhalis* infection in 24hrs (Smidt et al., 2013). Although this limits testing windows to just a few hours the results obtained from such tests are consistent and reproducible, which is why mice remain the model of choice for *Moraxella catarrhalis* infections.

Chinchillas are also used to study otitis media infections (Shaffer et al., 2013). Unlike mice which rapidly clear *Moraxella catarrhalis*, the nasopharynx of chinchillas is colonised for approximately one week by *Moraxella catarrhalis*. The main limitation to the chinchilla model is that not all of the animals challenged with *Moraxella catarrhalis* will develop otitis media. The main benefit to the chinchilla model is that a multispecies infection can be established, which better simulates a natural infection (Perez et al., 2014). This may enable a better understanding of how effective a potential vaccine will be, although only in the latter stages of research as multiple species also add another layer of variability to any results.

One solution to the lack of animal models is to use so called ‘humanized’ mice, which are immune-compromised mice that have been grafted with human immune cells or tissues. These mice can better serve as hosts for human specific pathogens such as

*Moraxella catarrhalis* and even produce some pathogen-specific human immune responses (Rämer et al., 2011).

### 1.5.2 Benefits

Much like the 23 valent pneumococcal and MenAWCY vaccines, any *Moraxella catarrhalis* vaccine will most probably be a multivalent vaccine containing a variety of surface expressed proteins. This would facilitate immune clearance and stimulate protection by blocking multiple pathogenicity targets.

Vaccination against *Moraxella catarrhalis* is rapidly becoming a necessity and has the potential to alleviate a massive socioeconomic burden. A *Moraxella catarrhalis* vaccine could prevent 10% of all COPD exacerbations and given the role that *Moraxella catarrhalis* plays as a co pathogen the results of a *Moraxella catarrhalis* vaccine could be much broader than expected.

## 1.6 Adhesion mechanisms

*Moraxella catarrhalis* colonises mucosal surfaces in both the upper and lower respiratory tract, colonisation is a multifactorial event with a range of proteins involved, both host and bacteria (de Vries et al., 2009).

#### 1.6.1.1 Type IV pili

Type IV pili (TFP) mediate long range interactions between *Moraxella catarrhalis* and host cells, these filamentous structures are made up of repeating units of *pilA* (de Vries

et al., 2009). The pili produced by *Moraxella catarrhalis* are highly conserved with little variation; only 2 antigenic variants of *pilA* are evident between strains (Luke-Marshall, Sauberan and Campagnari, 2011). TFP deficient mutants have reduced virulence due to lower rates of colonisation as well as impaired biofilm formation (Luke et al., 2007).

#### 1.6.1.2 McaP

McaP is a conventional autotransporter (see section 1.5.2) which displays adhesion, lipolytic, esterase and phospholipase B activity (Timpe et al., 2003). Knockouts of this 62kDa protein display reduced adhesion to A549 epithelial cells and lack any esterase activity. The adhesive domain of this protein is located in the 12 stranded beta-barrel N-terminal passenger region (Lipski et al., 2006).

#### 1.6.1.3 OMP CD

OMP CD is a porin like protein which adheres to middle ear mucin and A459 cells. This heat sensitive protein consists of a beta barrel anchor, a linker region and a C-terminal domain (Murphy, Kirkham and Lesse, 1993). Bioinformatics analysis demonstrates that both the beta barrel and linker regions are required for adhesion to A459 cells.

#### 1.6.1.4 Mha

The *Moraxella catarrhalis* filamentous haemagglutinin-like Mha proteins MhaC, MhaB1, and MhaB2 are part of a two-partner secretion system, whereby MhaC forms a porin like structure which facilitates the transport of the associated MhaB1 and MhaB2 proteins across the outer membrane. These proteins mediate the attachment to HEp2, Chang, and 16HBE14o− cells (Balder et al., 2007) (Guérin et al., 2017).

#### 1.6.1.5 Lipid oligosaccharides

Lipid oligosaccharides (LOS) are proteins which form much of the outer membrane of *M. catarrhalis*. There are 3 serotypes of LOS and they all have the same fundamental structure, a lipid A molecule connected to the oligosaccharide core via a kdo residue, only differing in their R groups (Holme et al., 1999). LOS A is the most prevalent serotype, found in 72% of strains, although most of these strains are isolated from children. LOS B and C are found in approximately 21% and 2% of strains respectively (Verhaegh et al., 2008). Although the exact mechanisms of adhesion are as yet unknown LOS deficient mutants such as O35E *lpxA* knockout results in reduced adhesion to Chang cells (Spaniol et al., 2008). The main role LOS has in adhesion appears to be due to membrane stability and integrity as a disrupted membrane affects the surface display of other adhesion molecules (de Vries et al., 2009).

### 1.6.2 Trimeric autotransporters

Trimeric autotransporter (TAA) proteins are virulence factors embedded in the outer membrane of some gram-negative bacteria (Cotter, Surana and St. Geme, 2005). They are part of the type V secretion system and have a unique tripartite structure consisting of an anchor, stalk and head region (Linke et al., 2006). Whilst there are some variations between different types of TAAs they all possess a left-handed beta roll anchor at the C terminus (Szczesny and Lupas, 2008). This region serves the dual purpose of translocating the stalk region and anchoring the protein complex to the outer membrane, essentially functioning as a porin within the outer membrane (Mikula et al., 2011). The general structure of the stalk domain is reminiscent of a rope, it is formed from alpha helical coiled coils (Linke et al., 2006). This region extends the head region away from the bacterial cell surface allowing it to make contact with the host extracellular matrix. There are various head domain configurations, the most common of which is the YadA-like head (Nummelin et al., 2004). It is a nine coiled left-handed beta roll comprised of trimeric single strand, left-handed beta-helices. This region functions as an adhesin and usually interacts with ECM components as well as mediating autoagglutination. *Moraxella catarrhalis* has 2 main types of TAAs MID/Hag and the UspA proteins (de Vries et al., 2009).

#### 1.6.2.1 MID/Hag

MID/Hag is a multifunctional trimeric autotransporter protein primarily involved in the binding to soluble IgD as well as acting as a B-lymphocyte mitogen, stimulating the production of IgD bearing B cells (Forsgren et al., 2001). MID/Hag has the classic trimeric autotransporter structure with a globular head domain, stalk region and beta

barrel translocator C-terminus. The N-terminal head domain mediates adherence to host receptors as well as IgD binding and hemagglutination (Pearson et al., 2002).

#### 1.6.2.2 UspA proteins

UspA are also trimeric autotransporters and although they have the general head-stalk-anchor configuration there are some notable differences. Whilst the anchor region has the same left- hand beta roll as other TAAs the head and stalk regions are distinct. The N-terminal head domain of the UspA proteins is a left-handed beta propeller structure, comprised a series of repeating residues (Hoiczyk, 2000). As with all TAAs the stalk region of UspA is comprised of alpha helical coiled coils, unlike the right-hand coiled YadA stalk the UspA stalk is an extended left-handed coiled coil which at 600-700A is approximately 400-500 angstroms longer than the YadA stalk (Agnew et al., 2011) (Conners et al., 2008) (Hoiczyk, 2000) [figure 2]. The *Moraxella catarrhalis* genome encodes for 2 distinct ubiquitous surface proteins; UspA1 and UspA2. Purified samples of these proteins are easily identified from one another via SDS-PAGE gel. Exposure to the heat and chaotropic agents required for SDS-PAGE gel separation causes UspA1 to dissociate resulting in a 100kDa monomeric protein (McMichael, Fiske and Fredenburg, 1998). This denaturation does not affect the CEACAM binding properties of UspA1 which indicates that the region responsible for CC1 binding is a specific sequence and not configuration dependant (Hill et al., 2005).

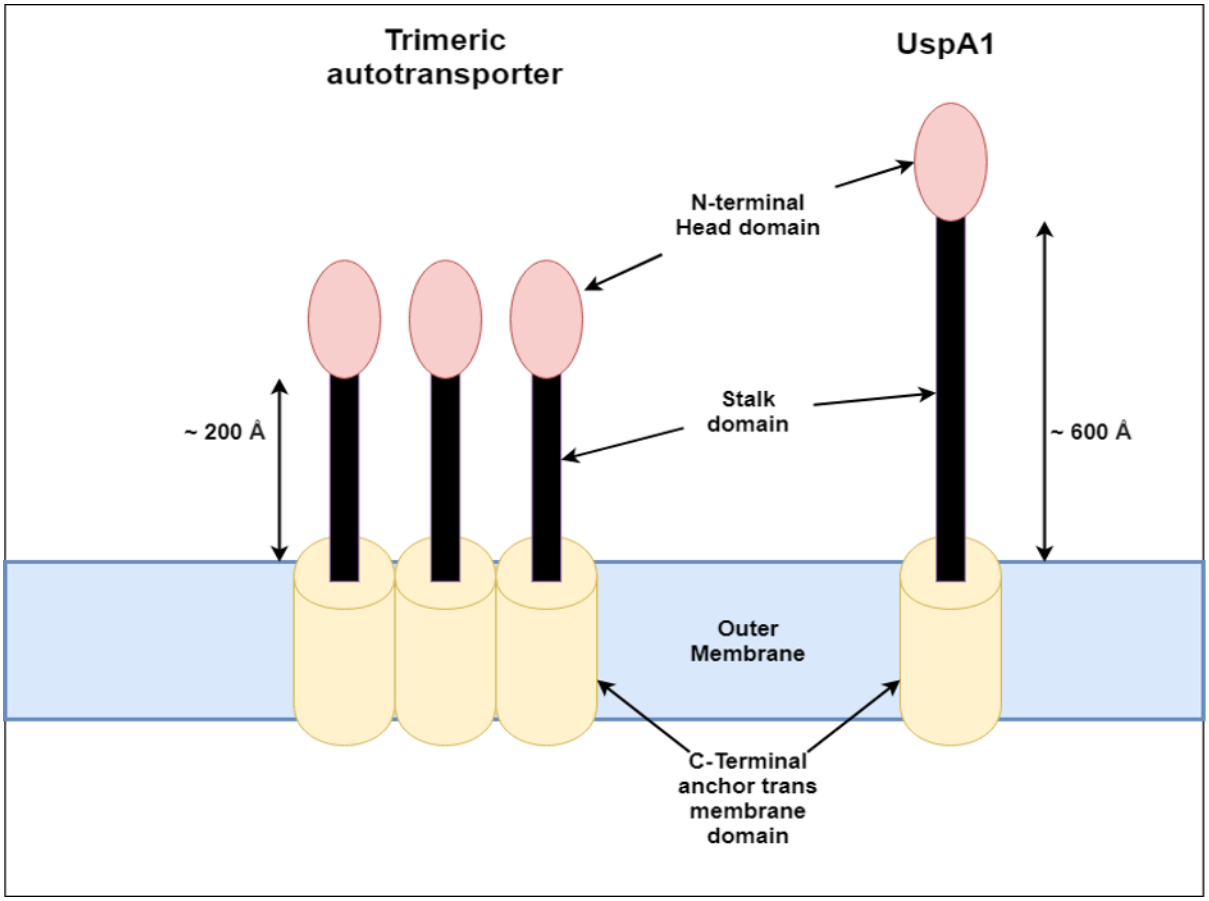



Figure 2 Trimeric autotransporters

Diagram comparing a full-length oligomer of a general Trimeric Autotransporter and an UspA1 monomer. This diagram highlights the disparty in length of the UspA1 stalk compaired to a standard trimeric autotransporter as well as the overall structure of both proteins.

Image adaptation based upon Conners et al. (2008)

#### 1.6.2.2.1 UspA1

UspA1 was the first of these proteins to be identified and functions mainly as an adhesin; binding fibronectin and laminin as well as carcinoembryonic antigen-related cell adhesion molecule 1 (CC1) (Hill and Virji, 2003) (Hill et al., 2005). Although it is mainly involved in host invasion UspA1 also targets complement related factors such as C3 and C4 binding protein. Whilst interactions with fibronectin and laminin are carried out by the head region, the CEACAM binding domain of UspA1 is found in the stalk. The CC1 binding domain was first identified in the MX2 strain of *Moraxella catarrhalis* and is located 400A from the tip of N terminus head in a region termed rD-7 (Hill et al., 2005). The CC1 binding properties of the UspA1 rD-7 region are heat stable indicating that the binding event is due to a specific amino acid sequence and not a folded conformation. This rD-7 region encompasses residues 527-655 and the CC1 binding motif is found within amino acids 578–597 (Conners et al., 2008).

UspA1 proteins are densely packed on the surface of *Moraxella catarrhalis* cells in a lollipop forest fashion which could make reaching the CC1 binding site difficult (Agnew et al., 2011). UspA1 proteins overcome this problem by introducing a weakness in the coiled coil stalk within the rD-7 region which allows it to bend in a boomerang like fashion making the binding site much more accessible to the receptor (Conners et al., 2008) [figure 3]. This flexing of the stalk is due to the interruption of the hydrophobic internal residues by polar histidine located at residues 573, 584 and 629 (Conners et al., 2008). Although it is a trimeric oligomer, only two out of the three UspA1 proteins bind to CC1 molecules. This is due to the 30-60° bend in the stalk which blocks the binding of a third CC1 molecule to one of the monomers.

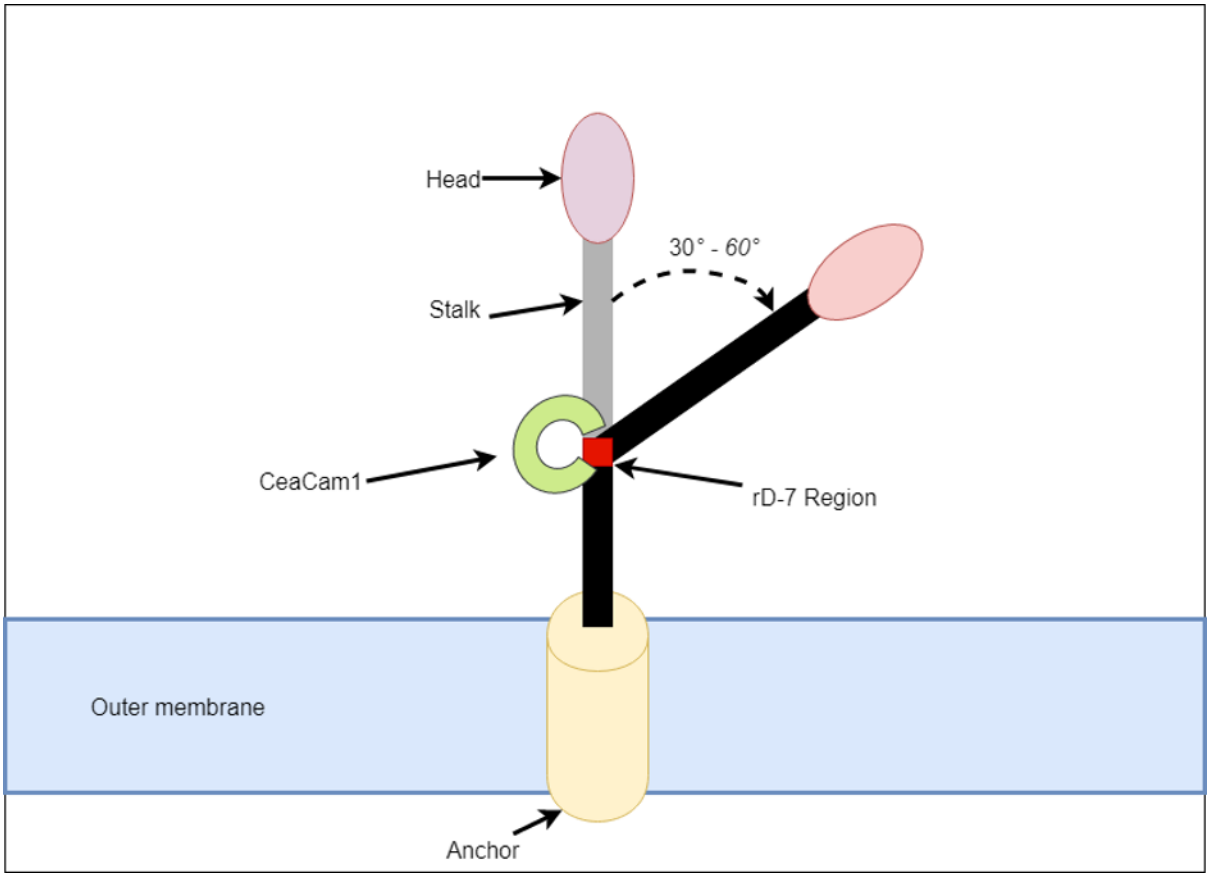



Figure 3 Schematic of UspA1 interaction with CC1.

This image shows the 30-60 degree bending of UspA1 to expose the CC1 binding rD-7 region, located at residues 586-628. Image adaptation based upon Conners et al. (2008)

#### 1.6.2.2.2 UspA2

Although UspA2 shares a structural identity of approximately 43% with UspA1 it does not share the CEACAM binding properties as it lacks the rD-7 region (Hill and Virji, 2003). UspA2 is predominantly involved with host immune evasion, preventing clearance of the bacteria via the complement cascade. UspA2 avoids complement mediated killing by binding a range of complement factors involved in the classical pathway including C3, C4bp and vitronectin (Nordström et al., 2004). The binding of vitronectin is a key determinant in whether a strain will be serum sensitive or serum resistant, this is because binding vitronectin interferes with the polymerisation of C9 which prevents the formation of the membrane attack complex (Attia et al., 2006).

Whilst both UspA proteins differ greatly, sequencing of the 035E genome has identified a 140 amino acid region of commonality where both genes are 93% identical (Lafontaine et al., 2000).

#### 1.6.2.2.3 UspA2H/V

The novel UspA proteins UspA2H and UspA2V have arisen either through inter- or intra- genetic homologous recombination (Hill, Whittles and Virji, 2012). The hybrid proteins have properties which can be attributed to both UspA1 and UspA2. UspA2H was the first of these novel adhesins to be discovered, its N-terminal identical to UspA1 whilst its C-terminal is derived from UspA2 (Hill and Virji, 2003) (Lafontaine et al., 2000). Due to the UspA1 head domain A2H can function as an adhesin. Recent studies have shown that approximately 20% of *Moraxella catarrhalis* strains possess an *UspA2H* gene instead of an *UspA2* gene (Lafontaine et al., 2000).

UspA2V is the second variant to be discovered, it can bind both CC1 and vitronectin (Hill, Whittles and Virji, 2012). All strains which possess the UspA2V protein belong to the serosensitive group and 16s ribosomal sequencing data places them in 2/ 3 phylogenetic group (Blakeway et al., 2017). The 2/3 group does not normally interact with CC1 and is the older of the two major evolutionary groups of *M. catarrhalis*, thus UspA2V may actually represent an intermediate evolutionary step (Earl et al., 2016) (Blakeway et al., 2017).

UspA2H and UspA2V may only be the first of these novel hybrid proteins to be discovered and more research is needed to assess the possibility of other combinations. These homologous recombination events have been observed and encouraged experimentally, whereby UspA2V was generated via the inter-strain recombination of MX2s *UspA1* gene and the genome of the rD-7 negative strain 035E (Hill, Whittles and Virji, 2012). This observation has important implications for the treatment of *Moraxella catarrhalis* as selective pressure may encourage the recombination events resulting in more virulent proteins which can function as both adhesins and immune evasion proteins.

## 1.7 CEACAM

### 1.7.1 Carcinoembryonic antigen family

Carcinoembryonic antigen molecules (CEA) are human glycoproteins which belong to the immunoglobulin superfamily. They were first discovered in the bile ducts of the liver and are often referred to as biliary glycoproteins. The CEA family encodes for 18 genes and 11 pseudogenes, which are found in a 1.2MB cluster on the long arm of chromosome 19 (Hammarström, 1999).

There are 2 subgroups of the carcinoembryonic antigen family: carcinoembryonic antigen related cell adhesion molecules (CEACAM) and pregnancy specific glycoproteins (PSG). CEACAMs are involved in cell adhesion and the PSGs are immunomodulators which protect the foetus during development (Lisboa et al., 2010) (Hammarström, 1999).

The CEACAM proteins are cell-cell adhesion molecules and can be detected on a range of cell types including epithelia, endothelial and leukocytes (Hammarström, 1999). CEACAM interactions are involved in many cellular processes including but not limited to; angiogenesis, differentiation, apoptosis and the modulation of immune responses (Tchoupa, Schuhmacher and Hauck, 2014) (Wagener and Ergün, 2000). Cell adhesion mediated by CEACAMs can be either a homophilic reaction, via CEACAM-Fc fusion protein, or a heterophilic reaction utilizing a CEACAM binding ligand such as UspA1 (Gray-Owen and Blumberg, 2006). Homophilic interactions are common between human cells whereas heterophilic reactions are usually between host cells and pathogens.

### 1.7.2 CEACAM1

CC1 originally called CD66a was the first of the CEACAM proteins to be discovered. It is primarily found on the apical surface of epithelial cells in the bile ducts, and the upper and lower respiratory tracts (GeneCards, 2019).

Each member of the CEACAM family has the same basic structure; an N-terminal immunoglobulin variable-region-like (IgV-like) domain, a variable number of immunoglobulin constant-region-type-2-like (IgC2-like) domains and a transmembrane anchor region (Villullas et al., 2007). The proteins are also heavily glycosylated, constituting over half of the proteins molecular weight (GeneCards, 2019) (Nagy and Senis, 2019).

There are 11 different isoforms of CC1 which are produced via the alternative splicing of the 9 exon regions of the *CC1* gene (GeneCards, 2019) [figure 4]. These isoforms differ extracellularly in the number of their IgC2-like domains and the level of glycosylation. With regards to the cytoplasmic domains there are 4 main variables that may arise; either a long or short cytoplasmic tail, a unique C-terminus which indicates that the protein will be secreted or an Alu repeat sequence in the open reading frame (Gray-Owen and Blumberg, 2006). The intercellular binding and the intracellular signalling properties of the isoforms vary depending on which immunoglobulin-region-type extracellular domains are present.

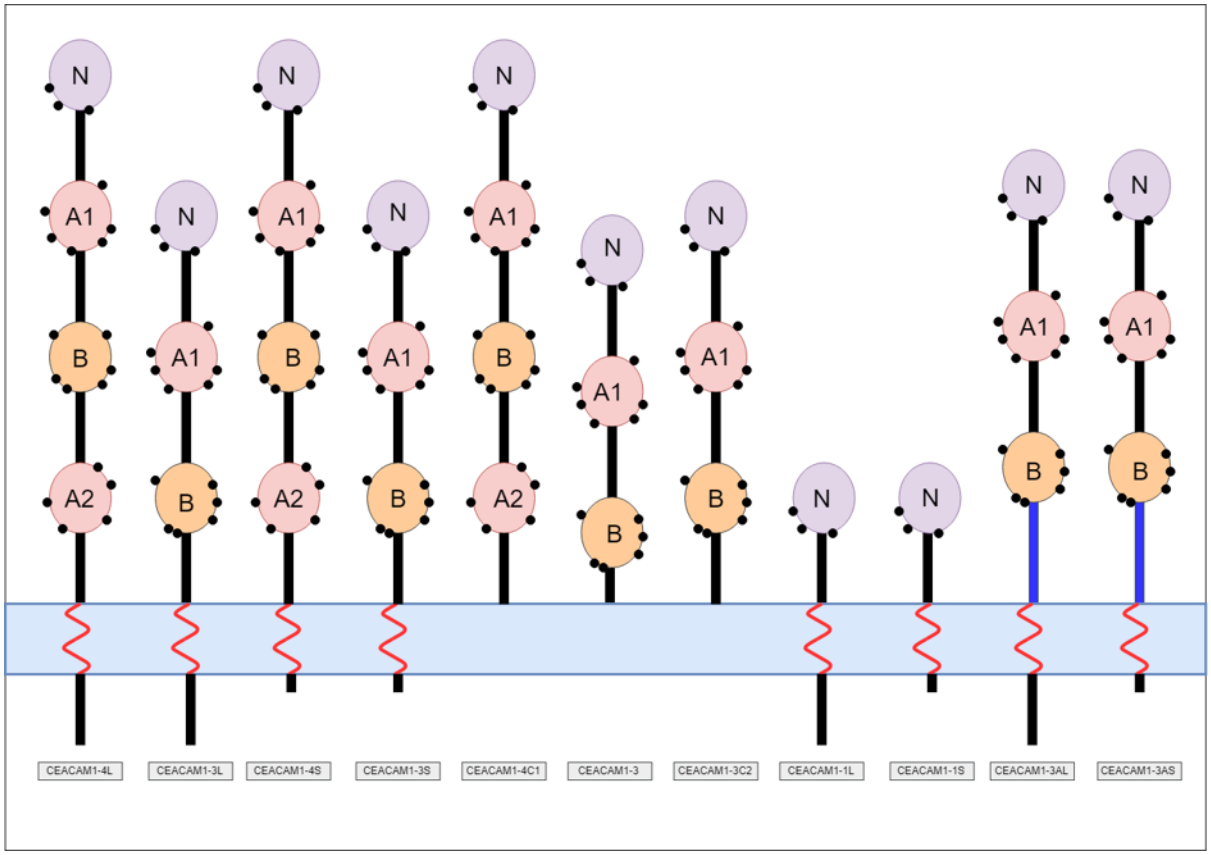



Figure 4 Isoforms of Human CC1.

Isoforms are generated via alternative splicing of CC1 transcripts in order to vary in the number of IgC2 domains and cytoplasmic tails. The heavy glycosaltion of the protiens is also evident, this results in CC1 appearing to have a much higher molecular weight when examined via SDS-Page gel.

Key: Amino-terminal IgV-like domain (purple circle). IgC2-like domain (red/orange circle). N-linked glycosylation site (black circle). Transmembrane domain (red zig zag). Alu sequence (blue line).

#### 1.7.2.1 CC1 and infection

The ubiquitous presence of CC1 on respiratory epithelial cells makes it an ideal target for pathogenic bacteria (Hill et al., 2005) (Tchoupa, Schuhmacher and Hauck, 2014). It is an important receptor for many bacterial adhesins such as the UspA1 protein of *Moraxella catarrhalis* as well as the OPA proteins of *Neisseria* (Hill et al., 2005) (Martin et al., 2016). As well as acting as a pathway to colonise host cells, CC1 binding has also been found to inhibit immune-cell function thus suppressing specific immune responses to infection (Gray-Owen and Blumberg, 2006).

During a *Moraxella catarrhalis* infection the CC1 binding ligand UspA1 flexes in the stalk region exposing the specific CC1 binding motif, the so called rD-7 region (Conners et al., 2008) (Hill et al., 2005). This region is a sequence specific receptor for CC1 meaning that even after denaturing it is still capable of binding CC1. Although UspA1 is a trimeric oligomer and each of the monomers has an rD-7 region it only binds two molecules of CC1 at a time, this is due to the bending of the UspA1 stalk. This conformational change results in two rD-7 regions being fully exposed for CC1 interactions whilst the third is inaccessible to the receptor site (Conners et al., 2008).

In contrast to the heat stable interactions between UspA1 and CC1, the OPA proteins of *Neisseria* and OMP P1 of *Haemophilus influenza* are labile. CC1 binding by OPA and OMP P1 is a structure dependant event (Tchoupa et al., 2015) (Martin et al., 2016). The 3-dimensional structure that the proteins form when folded brings otherwise physically distant motifs into proximity which together act as a ligand for CC1 (Virji et al., 1999).

# 2 AIMS

Screening of *Moraxella catarrhalis* isolates for CC1 binding activity revealed that the strain dubbed MX13L, a derivative of the parent strain ATCC25240, adhered to CC1. This is unexpected as this strain lacks expression of both UspA1 and UspA2. Further investigations confirmed that MX13L did not possess a functional rD-7 region, as anti-rD-7 antibodies did not bind to this strain.

Although SDS-Page gels and Western blots did not reveal any CC1 binding activity, dot blots of MX13L continued to demonstrate binding. This heat lability indicated that the novel ligand relied on a 3-dimensional structure to bind CEACAM. All other labile CC1 adhesins have a beta barrel structure, such as the *Neisseria* OPA proteins or the OMP P1 of *Haemophilus influenza* (Tchoupa et al., 2015) (Martin et al., 2016). This project aims to identify the novel CC1 binding ligand found in MX13L.

## 2.1 Value

In the last few decades, *Moraxella catarrhalis* has established itself as an important pathogen of the upper and lower respiratory tracts, along with rising incidences of sepsis (Verduin et al., 2002). It is a serious cause of morbidity and a drain on the healthcare system. Although antibiotic resistance is widespread in the *Moraxella catarrhalis* population with beta lactamases being obsolete and resistance to second generation cephalosporins rising, there is currently no vaccine (Shi et al., 2018) (Khan et al., 2009). Identifying a new CC1 binding ligand will not only provide a greater understanding of the pathogenicity of *Moraxella catarrhalis* but it may also provide a potential vaccine target.

## 2.2 Structure

Due to the labile nature of the ligand we were unable to identify the molecular weight of this protein. Instead the first step in investigating the adhesin was to create a target list using bioinformatic techniques. As all other examples of labile ligands are beta barrel structures a list of the beta barrel outer membrane proteins (BOMPs) found in ATCC25240 needed to be generated. Once a target list was obtained molecular biology techniques were employed. This included generating primers to the target BOMPs, with the aim of expressing the proteins in *E. coli* and performing adhesion assays on CC1 expressing HeLa cells. A transposon library was also generated in order to knockout the CC1 binding gene, confirming the results of the adhesion assays.

## 2.3 Objectives

This project aims to identify the novel CC1 binding ligand found in MX13L by:

- Creating a target list of potential beta barrel outer membrane proteins found in strain ATCC25240 using bioinformatic techniques
- Expressing all target proteins in *E.coli* via ligation independent cloning
- Assessing the CC1 adhesive properties of target proteins via adhesion assays
- Using the ez tn5 transposon to generate a transposon library in order to generate random genetic knock outs of Mx13L
- Identifying which randomly generated mutants of Mx13L did not bind to CC1 via dot blots
- Sending the knockouts for sequencing to identify which protein is the novel CC1 adhesin.

# 3 METHODS

## 3.1 Cell strains and growth conditions

### 3.1.1 Bacterial strains and growth conditions

*Moraxella catarrhalis* strains were grown either on heart brain infusion (BHI) agar or in heart brain infusion broth, both were supplemented with 10% (v/v) heated Horse blood. For transposon work HBHI plates were also supplemented with 50 $\mu g \cdot mL^{-1}$ kanamycin (Kan).

*E. coli* strains were grown either on Lysogeny Broth (LB) agar or broth, as *E. coli* were used to produce recombinant proteins the plates/ broth were often supplemented with antibiotics and compounds. Ampicillin (Amp) at a concentration of 100 $\mu g \cdot mL^{-1}$ as well as 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and 20 $\mu g \cdot mL^{-1}$ 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) were used in screening and inducing the production of prokaryotic recombinant proteins. Zeocin™ (Zeo) at a concentration of 100 $\mu g \cdot mL^{-1}$ replaced the ampicillin when inducing the production of eukaryotic proteins in XL10-Gold cells.

Both *Moraxella catarrhalis* strains and *E. coli* strains were grown for approximately 12-16 hours overnight at 37 °C in a 5% $CO_2$ incubator. Both static and shaking conditions were used for liquid cultures, shaking occurred at 200 RPM.

Stocks of bacteria were stored at -80 °C in the appropriate growth medium (LB broth for *E. coli* and HBHI for *Moraxella catarrhalis* strains), containing 25 % (v/v) glycerol.

### 3.1.2 Eukaryotic strains and growth conditions

All cells were grown in a forced air incubator at 37 °C with 5 % $CO_2$. HeLa cell lines used were grown in Roswell Park Memorial Institute medium 1640 (RPMI1640), whilst CHO cells were grown in Ham′s Nutrient Mixture F12. Both growth media were supplemented with 100 µg. $mL^{-1}$ penicillin (Pen); 100 µg.$mL^{-1}$ streptomycin (Strep); 300 µg.$mL^{-1}$ L-glutamine; and 10 % (v/v) foetal bovine serum (FBS). When low serum, < 5 % (v/v) FBS, or serum free conditions were required, FBS was replaced with Gibco® Insulin-Transferrin-Selenium (ITSG) media supplement.

Prior to use in experiments cells were passaged a minimum of three times to ensure proper growth and absence of any visible contamination. When cells reached ~80% confluency they were passaged via tryptic digestion. Growth media was discarded from the flask and cells were washed with 5 mL of Dulbecco's phosphate buffered saline (DPBS) to remove any residual traces of FBS. The DPBS was aspirated and cells were incubated at 37°C for 5 minutes with 5 mL of 0.25% trypsin-EDTA ($2.5x10^3$ µg.$mL^{-1}$ Trypsin and 200 µg.$mL^{-1}$ of EDTA) (Ethylenediaminetetraacetic acid). Once cells had detached 5 mL of the appropriate media was added in order to neutralise the protease activity of trypsin. Cells were then centrifuged at 300 g for 5 minutes. Residual media was discarded, and the pellet resuspended in growth media. Cells were then reseeded into a vent cap cell culture flask containing the appropriate growth media.

### 3.1.3 Freezing eukaryotic cells

To store eukaryotic cells long term the cells were kept in liquid nitrogen below -135 °C. In preparation for storage, cells were passaged via tryptic digestion as normal. The growth media was discarded from the flask and 5 mL of DPBS was washed over the cell. After the DPBS was removed, cells were incubated at 37 °C for 5 minutes with 5 mL of 0.25% trypsin-EDTA. When 80-100% of the cells had detached from the flask surface 5ml of growth media was added to neutralise the trypsin activity. Cells were then centrifuged at 300 g for 5 minutes. Residual media was discarded, and the pellet resuspended in freezing media which consisted of 90% FBS + 10% DMSO (Dimethyl sulfoxide). Working quickly the resuspended cells were aliquoted into cryovials before being placed into a controlled freezing container which submerged the cells in isopropanol. This freezing chamber was then placed into a -80 °C freezer overnight whereby the container cooled the cells at a rate of -1 °C per minute. The next day cells were removed from the freezing chamber and deposited into liquid nitrogen for long term storage.

## 3.2 Producing recombinant CC1-Fc

### 3.2.1 Creating a stable CC1-Fc secreting cell line

#### 3.2.1.1 Selecting a plasmid

To produce recombinant soluble CC1-Fc proteins, the gene encoding residues for extracellular protein, were cloned into pINFUSE-hIgG2-Fc2 using ligation independent cloning. The pINFUSE family of plasmids feature Fc regions with introns, which

enhances the levels of eukaryotic gene expressions. pINFUSE-hIgG2-Fc2 is a cloning plasmid specifically designed to generate a secreted Fc-fusion protein. To accomplish this the plasmid expresses the CH2 and CH3 domains, of the Fc region, of human IgG2 heavy chain, as well as the hinge region. In addition to this pINFUSE-hIgG2-Fc2 also encodes the IL2 signal sequence allowing Fc-Fusion proteins to be generated from proteins that are not naturally secreted.

#### 3.2.1.2 Amplifying CC1-Fc

CC1-Fc encoding DNA was amplified using polymerase chain reaction (PCR) using primers (appendix 1) designed to amplify the shorter isoform from extracted full length CC1-4L DNA. The primers also contained 5' DNA regions homologous to linear pINFUSE-hIgG2-Fc2 in order to facilitate ligation independent cloning. PCR mix was as follows: 7 µL nuclease free water ($ddH_2O$), 0.5 µM of forward and reverse primers, as well as 1 $ng.\mu L^{-1}$ genomic DNA were added to 10 µL CloneAmp™ high fidelity PCR premix in a 0.2 mL PCR tube. PCR was performed under the following conditions: initial denaturing 95 °C 5 min; followed by 30 cycles of: 95 °C 15 sec, 60 °C 15 sec, 72 °C 5 sec/kbase DNA; finished by 72 °C 10 min for the final extension.

#### 3.2.1.3 XL10-Gold cell transformation

The pINFUSE-hIgG2-Fc2 plasmid was linearized via digestion at 37 °C for 1 hr by the restriction enzymes *Bgl*II-HF® and *EcoR*V-HF®. The linearized plasmid was then purified using QIAquick® PCR Purification Kit according to the manufacturer's

instructions in order to remove the restriction enzymes. After purification a 0.8% agarose gel comparing the linearized plasmid and uncut plasmid was run at 200 V for 1 hour in order to ensure that the plasmid had been fully linearized.

A 2:1 molar ratio of amplified CC1-Fc and linear pINFUSE-hIgG2-Fc2 were incubated together at 50 °C for 15 min with 1X In-Fusion® master mix. This reaction mix was then incubated on ice for 30 min with chemically competent XL10-Gold ultracompetent cells. In order to induce the uptake of the vector the cells were heat-shocked by incubation at 42 °C for 40 secs followed by 5 min on ice. SOC medium (Super Optimal broth with Catabolite repression) preheated to 37 °C was added to promote the recovery of the XL10-Gold ultracompetent cells as well as boost the uptake of plasmid. Following a recovery period of 1 hour in a shaking incubator at 200 RPM and 37 °C, transformed cells were streaked onto selection plates (see section 3.1.1) and incubated overnight at 37 °C.

As pINFUSE-hIgG2-Fc2 did not contain a lac operon blue white screening was not possible, thus individual colonies were picked at random and grown in 10 mL of selection LB broth overnight in the shaking incubator with the previously described conditions. Plasmid DNA was extracted via a QIAprep® Spin kit and the concentration of the plasmid was adjusted to 100 $ng \cdot \mu L^{-1}$ using a UV-Vis spectrophotometer. PCR was used to confirm successful transformation using the same method described above.

#### 3.2.1.4 Kill curve

Prior to transfection with CC1-Fc DNA a kill curve was undertaken to determine the optimum concentration of antibiotic, in this case Zeocin, required to kill all

untransfected CHO cells. Cells were seeded in a 24 well tissue culture plate with F-12 Ham media and allowed to grow until 80% confluency. Media was then aspirated and replaced with F-12 Ham supplemented with zeocin at concentrations ranging from 1000 $\mu g \cdot mL^{-1}$ to 0 $\mu g \cdot mL^{-1}$. For 10 days the wells were examined daily, and the media replaced every 2 days to ensure that the concentration of antibiotic remained constant. After 10 days the minimum Zeocin concentration required to kill all cells was determined.

#### 3.2.1.5 Transfecting CHO cells

Prior to transfection pINFUSE-hIgG2-Fc2-CC1-Fc DNA was linearized via digestion with *Not*I restriction enzyme using the same method as previously described (see section 3.2.3). Linear plasmids integrate better into eukaryotic genomes for stable transfection.

CHO cells were grown in 24 well tissue culture plates to 70-90% confluency. The cells were washed with 15 mL of Dulbecco's phosphate buffered saline (DPBS) to remove excess serum. Lipofectamine® 3000 transfection mixture was prepared in 2 tubes. In the first tube 36 µL lipofectamine 3000 reagent was diluted with 600 µL Opti MEM medium. The second tube mixed 24 µL of linearized pINFUSE-hIgG2-Fc2-CC1-Fc DNA with 48 µL of P3000 reagent and diluted this mixture with 1200 µL of Opti-MEM. After mixing the contents of the tubes in a 1:1 ratio, the Lipofectamine® 3000 transfection mixture was incubated for 15 min at room temperature. This incubation time allowed lipid complexes to form around the plasmid which ensured that the target DNA could be taken up by the CHO cells. CHO cells were inoculated with 50 µL of

Lipofectamine® 3000 transfection mixture per well and incubated for 24 hours at 37°C. After 24 hours 500 µL of Ham′s Nutrient Mixture F12 additionally supplemented with 400 $\mu g.mL^{-1}$ of Zeocin (as determined by kill curve) was added to each well.

#### 3.2.1.6 Determining CC1-Fc production post-transfection

In order to determine that transfected cells were successfully producing the recombinant protein the supernatant was collected and tested. A sample of supernatant was collected subjected to serial dilution with fresh media. 50 µL aliquots were transferred to a nitrocellulose membrane via direct pipetting and vacuum drying. Nitrocellulose was submerged in 3% (w/v) bovine serum albumin (BSA; diluted in PBS-T), for 1hr at room temperature, in order to block non-specific binding sites. 3% BSA was then aspirated and the nitrocellulose strip overlaid with A0115 diluted from stock solutions to 1 $\mu g.mL^{-1}$ in 1% (w/v) BSA in PBS-T with 0.05% (w/v) $NaN_3$ at RT for 1 hour. The membrane was then washed 3 times with PBS-T and the final wash was incubated for 5 min before being aspirated. The secondary antibody applied was anti-rabbit-Fc conjugated to alkaline phosphatase diluted to 1 $\mu g.mL^{-1}$ in 1% (w/v) BSA in PBS-T with 0.05% (w/v) $NaN_3$ at RT for 1 hour. The wash step was repeated as before. The blot was developed for 15 mins using 6.6 $\mu L \cdot mL^{-1}$ of NBT and 3.3 $\mu L \cdot mL^{-1}$ of BCIP in AP buffer.

### 3.2.1.7 Monoclonal cell line selection

The wells which resulted in positive supernatants underwent tryptic digestion; half of the cells were reseeded into a T-175 tissue culture flask to maintain a polyclonal population. The other half of the CC1-Fc transfected CHO cells were seeded into a 96 well tissue culture plate via limiting dilution, in order to select a monoclonal population. Both sets of cells were maintained in Ham's Nutrient Mixture F12 containing 400 $\mu g.mL^{-1}$ of zeocin.

Cells undergoing monoclonal selection had their growth media replaced every 48 hours. After 11 days under selection pressure any monoclonal populations evident were identified, and their populations expanded via growth in separate T-75 tissue culture flasks.

### 3.2.1.8 Determining the production of CC1-Fc in monoclonal populations

In order to ensure that the selected monoclonal populations were producing CC1-Fc, 50 µL aliquots of supernatant from each population, were transferred to a nitrocellulose membrane. Nitrocellulose was submerged in 3% (w/v) BSA, diluted in PBS-T, for 1 hour at room temperature, to block non-specific binding sites. The BSA was removed and the nitrocellulose strip was then overlaid with A0115 diluted from stock solutions to 1 $\mu g.mL^{-1}$ in 1% (w/v) BSA in PBS-T with 0.05% (w/v) $NaN_3$ at RT for 1 hour. The membrane was washed 3 times with PBS-T and the final wash was incubated for 5 min before being aspirated. The secondary antibody was applied at RT for 1 hour; anti-rabbit-Fc conjugated to alkaline phosphatase diluted to 1 $\mu g.mL^{-1}$ in 1% (w/v) BSA in PBS-T with 0.05% (w/v) $NaN_3$. The wash step was repeated as before. The blots

were developed for 15 mins using 6.6 $\mu L \cdot mL^{-1}$ of NBT and 3.3 $\mu L \cdot mL^{-1}$ of BCIP in AP buffer.

#### 3.2.1.9 Protein Purification

The supernatants collected from both monoclonal and polyclonal populations were pooled and filtered through a 0.2 µm syringe filter to remove any cell debris. Prior to protein purification a gravity flow column containing 1 mL Protein A-Sepharose® resin was equilibrated by washing 15 column volumes (CV) of protein A loading buffer through the column. The supernatant was then mixed with Protein A Loading Buffer in a 1:1 ratio before being added to the column and allowed to flow through under gravity. A further 15 CV of loading buffer were passed through the column to ensure any unbound proteins were removed. 3 CV of elution buffer were passed through the column, and fractions were collected and immediately neutralised with Protein A Neutralisation Buffer. The collected fractions were purified via dialysis; fractions were loaded into dialysis tubing membrane 5000 kDa MWCO and placed into 5 L of dialysis buffer. Dialysis occurred overnight and the buffer was changed 3 times with the last change left overnight. The total volume was concentrated using Vivaspin® 20 spin columns and final concentration was determined via the Pierce™ BCA Protein Assay Kit as per the manufacturer's instructions.

### 3.2.2 Determining the fidelity of CC1-Fc

#### 3.2.2.1 Dot Blot

To compare the interactions of the new recombinant CC1-Fc to a previously purchased laboratory stock of CC1-Fc (oCC1-Fc), 50 µL of *Moraxella catarrhalis* isolates grown to $OD_{280}$ 0.5 were transferred to nitrocellulose membrane via direct pipetting and vacuum drying. The non-specific binding sites were blocked for 1 hour at room temperature via 3% (w/v) bovine serum albumin (BSA; diluted in PBS-T). One membrane was overlaid with the recombinant CC1-Fc produced by the transfected CHO cells and the other with stock oCC1-Fc both diluted to 0.2 $\mu g \cdot mL^{-1}$ in 1% BSA-PBST. The membranes were washed twice with PBS-T, the third wash was incubated for 5 min for before being decanted. Anti-human-Fc conjugated to alkaline phosphatase diluted to 1 $\mu g.mL^{-1}$ in 1% (w/v) BSA in PBS-T with 0.05% (w/v) NaN3 was added to each strip and incubated for 1 hour at room temperature. The blots were washed as before and developed for 15 mins using developing buffer (6.6 $\mu L \cdot mL^{-1}$ of NBT and 3.3 $\mu L \cdot mL^{-1}$ of BCIP in AP buffer).

#### 3.2.2.2 SDS-Page gel & Western blot

To ensure that the recombinant protein produced was identical in function to oCC1-Fc it was necessary compare the size and functionality of CC1-Fc. A 7.5% resolving gel and a 5% stacking gel were prepared. The resolving gel consisted of 4.9 mL dH2O, 2.5 mL Tris hydrochloride (Tris HCl) pH 8.8, 100 µL 10% sodium dodecyl sulphate (SDS) 2.5 mL acrylamide. Whilst the stacking gel was made using 3.05 mL of dH2O, 1.25 mL Tris HCl pH 6.8, 50 µL 19% SDS, 650 µL acrylamide. Immediately prior to

pouring 50 µL 10% ammonium persulfate (APS) and 10 µL Tetramethyl ethylenediamine (TEMED) were added to the resolving gel whilst half that amount was added to the stacking gel. The resolving gel was poured and set before the stacking gel was layered on top. Once both gels had set, CC1-Fc and oCC1-Fc were standardised to 0.2 $\mu g \cdot mL^{-1}$ and boiled with to 20 µL Laemmli buffer at 95 °C for 5 mins. 10 µL of denatured samples were then loaded into each well. Gels were run in 1x SDS running buffer for 1.5 hours at 200 V.

One gel was stained with Coomassie in order to visualise the protein size, whilst the other was transferred onto nitrocellulose membrane via a wet transfer. To transfer the gel onto a nitrocellulose membrane both the gel and membrane were equilibrated in transfer buffer before being sandwiched together in a filter paper-gel-membrane-filter paper manner. The 'transfer sandwich' was clamped together by a support grid and placed into an electro transfer tank filled with transfer buffer for 1 hour at 100 V. A cooling block was also placed into the tank to dissipate the heat produced.

Once the gel had transferred the membrane was blocked with 3% (w/v) bovine serum albumin (BSA; diluted in PBS-T) for 1 hour at room temperature. The membrane was then incubated with 1 $\mu g.mL^{-1}$ A0115 in 1% (w/v) BSA in PBS-T with 0.05% (w/v) $NaN_3$ at for 1 hour at room temperature. The membrane was then washed 3 times with PBS-T, the final wash included a 5 min incubation period. AP conjugated anti-rabbit secondary antibody 1 $\mu g.mL^{-1}$ in 1% (w/v) BSA in PBS-T with 0.05% (w/v) $NaN_3$ was applied to the membrane for 1 hour at room temperature. The washing step was then repeated before the membrane was visualised with 6.6 $\mu L \cdot mL^{-1}$ of NBT and 3.3 $\mu L \cdot mL^{-1}$ of BCIP in AP buffer and the membrane left to develop for 15 minutes.

## 3.3 Identifying a novel CC1 binding protein

### 3.3.1 Assessing MX13L interactions with CC1

#### 3.3.1.1 Dot blot

To compare the interactions of different *Moraxella catarrhalis* strains with CC1-Fc, strains of *Moraxella catarrhalis* (see table) were grown and killed via freeze thaw lysis. The samples were standardized to $A_{280}$ OD 0.5 and 50 µL of each was transferred to nitrocellulose membrane via direct pipetting and vacuum drying. Using 3% (w/v) bovine serum albumin (BSA; diluted in PBS-T) the non-specific binding sites were blocked for 1hr at room temperature. After initial blocking CC1-Fc was diluted to 0.2 $\mu g \cdot mL^{-1}$ in 1% (w/v) BSA-PBST, this acted as the primary antibody, the membrane was soaked in primary antibody for 1 hour at room temperature. The membrane was then washed three times with PBS-T, the third wash was incubated for 5 min for before being decanted. Anti-human-Fc conjugated to alkaline phosphatase diluted to 1 $\mu g.mL^{-1}$ in 1% (w/v) BSA in PBS-T with 0.05% (w/v) $NaN_3$ was added to each strip and incubated for 1 hour at room temperature. The blots were washed as before and developed for 15 mins using developing buffer (6.6 $\mu L \cdot mL^{-1}$ of NBT and 3.3 $\mu L \cdot mL^{-1}$ of BCIP in AP buffer).

#### 3.3.1.2 Amplifying UspA1/ UspA2 proteins

In order to identify how MX13L was binding to CC1, PCR was performed to amplify UspA1 and UspA2 encoding DNA from the MX13L genome using the UspA specific primers (appendix 1). Due to the similarity of the various UspA2 type proteins, UspA2

primers would amplify UspA2/V/H (Hill, Whittles and Virji, 2012). MX2 and MX1 acted as positive controls for this experiment, MX2 encodes for UspA1 whilst MX1 encodes for UspA2V. PCR mix was as follows: 7µL nuclease free water ($ddH_2O$), 0.5 µM of forward and reverse primers, as well as 1 $ng.\mu L^{-1}$ genomic DNA were added to 10 µL CloneAmp™ high fidelity PCR premix in a 0.2 mL PCR tube. PCR was performed under the following conditions: initial denaturing 95 °C 5 min; followed by 30 cycles of: 95 °C 15 sec, 55 °C 15 sec, 72 °C 5 sec/kbase DNA; finished by 72 °C 10 min for the final extension.

#### 3.3.1.3 Determining rD-7 production

To ensure that the binding of MX13L to CC1 was not caused by presence of rD-7 within UspA1 or elsewhere, *Moraxella catarrhalis* strains; MX13L, MX13D, MX13ΔUsp, and MX2 were grown and lysed via freeze thaw lysis to release internal proteins. The bacteria lysates and purified rD-7 were standardized to $A_{280}$ OD 0.5 and 50 µL of each was transferred to nitrocellulose membrane via direct pipetting and vacuum drying. Using 3% (w/v) bovine serum albumin (BSA; diluted in PBS-T) the non-specific binding sites were blocked for 1 hour at room temperature. After initial block the membrane was overlaid with anti-rD-7 bleed-out diluted 1:100 in 1% (w/v) BSA-PBST for 1 hour at room temperature. The membrane was then washed three times with PBS-T, the third wash was incubated for 5 min before being decanted. Anti-rabbit-Fc conjugated to alkaline phosphatase diluted to 1 $\mu g.mL^{-1}$ in 1% (w/v) BSA in PBS-T with 0.05% (w/v) NaN3 was added to each strip and incubated for 1 hour at room temperature. The blots were washed as before and developed for 15 mins using developing buffer (6.6 $\mu L \cdot mL^{-1}$ of NBT and 3.3 $\mu L \cdot mL^{-1}$ of BCIP in AP buffer).

### 3.3.1.4 Western blot

A western blot was run with *Moraxella catarrhalis* strains; MX13L, MX13D, ATCC25240, and MX2, the OpaLess *Neisseria* strain was also utilised, to ascertain the molecular weight of the novel adhesin. A 7.5% resolving gel; 4.9 mL dH2O, 2.5 mL Tris HCl pH 8.8, 100 µL 10% SDS and 2.5 mL acrylamide. A 5% stacking gel was also prepared using 3.05 mL of dH2O, 1.25 mL Tris HCl pH 6.8, 50 µL 19% SDS and 650 µL acrylamide. Before the gels were poured 50 µL 10% APS and 10 µL TEMED were added to the resolving gel, whilst 25 µL 10% APS and 5 µL TEMED added to the stacking gel. The resolving gel was poured and set before the stacking gel was layered on top. Once both gels had set, the samples were boiled in 20 µL laemmli buffer at 95 °C for 5 mins. 10 µL of denatured samples were then loaded into each well. Gels were run in 1x SDS running buffer for 1.5 hours at 200 V.

To transfer the gel onto a nitrocellulose membrane both the gel and membrane were equilibrated in transfer buffer before being sandwiched between filter paper and held together by a support grid. The gel/membrane sandwich was then placed into a transfer buffer filled tank for 1 hour at 100 V. A cooling block was also placed into the tank to dissipate the heat produced.

Once the gel had transferred the membrane was blocked with 3% (w/v) bovine serum albumin (BSA; diluted in PBS-T) for 1 hour at room temperature. The membrane was then incubated with 1 $\mu g.mL^{-1}$ CC1-Fc in 1% (w/v) BSA in PBS-T with 0.05% (w/v) $NaN_3$ at for 1 hour at room temperature. The membrane was then washed 3 times with PBS-T, including a 5 min final wash. 1 $\mu g.mL^{-1}$ Rabbit-anti-Human-IgG(Fc)-AP secondary antibody was applied to the membrane for 1 hour at room temperature. The washing step was then repeated before the membrane was visualised with 6.6 $\mu L \cdot mL^{-1}$

of NBT and 3.3 $\mu L \cdot mL^{-1}$ of BCIP in AP buffer and the membrane left to develop for 15 minutes.

### 3.3.2 Identifying target proteins

#### 3.3.2.1 Bioinformatic analysis

The whole genome sequence of ATCC25240, parent strain of MX13L, was analysed by PSORT, a programme which determines the subcellular localisations of proteins. The results of the PSORT analysis was passed onto another programme called BOMP which can determine if a protein is likely to be a beta barrel or not.

#### 3.3.2.2 Amplifying target genes

In order to express the target BOMP proteins as faithfully to native as possible, stellar *E. coli* cells were transformed with the plasmid pOAF containing individual target protein encoding genes. pOAF is a surface expression vector based on the pOPINF plasmid; the *E. coli* OmpA signal peptide is encoded upstream of the pOPINF 5' add on sequence which facilitates surface expression of recombinant proteins on *E. coli* host strains.

The target genes were amplified via PCR from extracted MX13L DNA using primers designed with homologous 5' DNA overlap regions with linear pOAF (Table1). 7µL nuclease free water ($ddH_2O$), 0.5 µM of forward and reverse primers, as well as 1 $ng.\mu L^{-1}$ genomic DNA were added to 10 µL CloneAmp™ high fidelity PCR premix in a 0.2 mL PCR tube to form the PCR mix.

PCR was performed under the following conditions: initial denaturing 98 °C 5 min; followed by 30 cycles of: denaturing 98 °C 10 sec, annealing X °C 15 sec, extension 72 °C 5 sec·kBase$^{-1}$ DNA; finished by 72 °C 5 min for the final extension. The annealing temperature of the primers varied (table 1).

Table 1 Primers designed to bind to outer membrane beta barrel proteins in MX13 (ATCC25240).

The accession numbers of each target protein and the corresponding forward and reverse primers are listed as well as the required annealing temperature. The pOAF overlap sequences indicated at the bottom of the table were added to the 5' end of each primer to ensure that ligation independent cloning was possible.

| Accession ID | Name | Forward Primer 5'-3' | Reverse Primer 5'-3' | Annealing Temp (°C) |
|---|---|---|---|---|
| WP_003666550 | BOMP 1 | ATGAAAAAAACTTCCACACAG | TTAAAATGAAAAGCCAGTTG | 49 |
| WP_038519905 | BOMP 2 | ATGAATAAGTTTCAATTATTAC | TTAGTAAGTGAAGTTCACGCC | 52 |
| WP_003673045 | BOMP 3 | ATGAACCAAATTTTTCATTTG | CTACCACTTATAAGTCATTG | 46 |
| WP_038520226 | BOMP 4 | ATGAAAAAACTTGCTCTAGC | TTAGAATTTATATTCTAAACC | 49 |
| WP_003659887 | BOMP 5 | ATGAACATAAAAACTTTCAGC | TTAAAAATGATAATTTAGACC | 47 |
| WP_038519791 | BOMP 6 | ATGAATCAATCAAAACAAAAC | TTAAAACTTCATTTCAAGTGC | 47 |
| WP_038520099 | BOMP 7 | ATGTCAAAATCTATCACAAAAAC | TTAAAACTTCATTTCAAGACTG | 48 |
| WP_003661028 | BOMP 8 | ATGAAACCATCAATCATCAAAAAC | TTAGAAAATGAATGACACGCC | 51 |
| WP_003659692 | BOMP 9 | ATGAGCTTAAAATTTGGATAC | TTATTTGGCGTGATAAGCAAG | 51 |
| WP_003660701 | BOMP 10 | ATGCGTAATTCATATTTTAAAGG | TTAAAAGACACTACCAATCTGG | 50 |
| WP_038520205 | BOMP 11 | ATGAAAAAATTAATTTTAGCAAC | TTAAAAGTTATGGCGAAGAC | 48 |
| WP_038518786 | BOMP 12 | ATGATAAAAAAACCACTTGTTTTG | TCAAAATTTAGCCGTCAAACC | 52 |
| WP_003667703 | BOMP 13 | ATGAAGGTTACCATGATAAAAAAAC | TCAAAATTTGAAATTTGCCC | 50 |
| WP_038519721 | BOMP 14 | ATGCGTTTTATACCTTTTGC | TTAAAACTCAATGCTGGCAC | 51 |
| WP_003669513 | BOMP 15 | ATGCACACCGCTCATCACC | TTAGTAATCATGTCTCCAAGC | 58 |
| WP_038519603 | BOMP 16 | ATGAGTTTTTTAAAAAAGAAAAAG | TTAAGTGATTAAAAGAACATC | 45 |
| WP_038519295 | BOMP 17 | ATGTCAAAGCCCGTTTTGTTTG | TTAAAATGGTGTGCCAATAAAAAAATG | 55 |
| **pOAF 5' addition** | | **AAGTTCTGTTTCAGGGCCCG** | **ATGGTCTAGAAAGCTTTA** | |

#### 3.3.2.3 *E. coli* transformation

The plasmid was linearized via digestion at 37 °C for 1 hour by the restriction enzymes *Hind*III-HF® and *Kpn*I-HF®. The linearized plasmid was subsequently purified using QIAquick® PCR Purification Kit according to the manufacturer's instructions in order to remove the restriction enzymes.

The amplified target DNA was purified using the QIAquick PCR Purification Kit and cloned into pOAF using ligation independent cloning. Amplified target DNA and linear pOAF were incubated together in a 2:1 molar ratio at 50 °C for 15 min with 1X In-Fusion® master mix. This reaction mix was then incubated on ice for 30 min with chemically competent Stellar *E. coli* cells. Cells were heat-shocked by incubation at 42 °C for 40 secs followed by 5 min on ice to induce the uptake of cloned plasmids. to promote the recovery of the competent cells SOC medium preheated to 37 °C was added to the cells. Following a 1 hr recovery period in a 37 °C shaking incubator at 200 RPM, transformed cells were plated onto LB agar (containing Amp, IPTG and X-Gal) and incubated overnight at 37 °C.

Following overnight incubation on selection agar, colonies were selected, and transformation was confirmed via PCR as described above. Liquid cultures of successful transformations were set up overnight in selection LB broth (containing Amp, IPTG and X-Gal) at 37 °C (see 3.1.1 for more info). Glycerol stocks of each target protein were set up.

### 3.3.2.4 Adhesion assays

#### 3.3.2.4.1 Eukaryotic cell preparation

Prior to use in adhesion assay experiments HeLa cells expressing human CC1 and HeLa Neo cells that didn't express any CEACAM were grown to confluence at 37 °C in T-75 tissue culture flasks. The cells were passaged via tryptic digestion and 100 µL seeded into 96-well plates at a concentration of approximately $2x10^4$ cells per well. After 48 hrs cells had reached confluency and the media was replaced with Medium 199 (devoid of any antibiotics) pre-warmed to 37 °C.

#### 3.3.2.4.2 Bacterial preparation

Prior to use in adhesion assay experiments stellar *E. coli* transformed to surface express target proteins were grown overnight at 37 °C in LB broth supplemented with 1 mM IPTG and 100 $\mu g.mL^{-1}$ of AMP. The cultures were diluted to $A_{260}$ OD 1.0 in pre-warmed Medium 199 (devoid of any antibiotics), this optical density corresponded to an estimated multiplicity of infection (MOI) of 100 bacterial cells per 1 eukaryotic cell. Stellar *E. coli* expressing pOAF only, were also grown to the same conditions, these cells acted as a negative control.

#### 3.3.2.4.3 Infection and cell fixation

The media was removed from the eukaryotic cells, washed in antibiotic free media to remove antibiotics prior to bacterial overlay. 100 µL bacterial suspensions containing approximately $10^8$ per mL were added to each well. The plates were then incubated

at 37 °C for 3 hours. After incubation the wells were thoroughly washed with Medium 199 to remove unbound bacteria and then washed twice more with warm DPBS to remove any traces of media. Prior to the addition of a fixing agent all liquid was completely removed from the wells. To fix the cells 50 µL of 4% (w/v) paraformaldehyde (PFA; dissolved in PBS) was added to each well and the plates were incubated overnight at 4 °C.

#### 3.3.2.4.4 Cell staining

In order to prevent interference prior to staining PFA was removed and wells were washed with DPBS containing 0.05% [w/v] Sodium Azide, which was then discarded. To prevent non-specific antibody binding 100 µL of 3% (w/v) BSA was added to each well and the plates were incubated for 1 hour at room temperature on a rocker. The BSA was removed and 100 µL of the primary antibody was applied; 1 $\mu g.mL^{-1}$ of mouse anti-*E. coli* LPS in 1% (w/v) BSA in PBS-T. Cells were again incubated for 1 hour at room temperature on a rocker. The primary antibody was aspirated, and wells were washed 3 times with PBS-T, the third wash included a 5 min incubation time to ensure any antibody residue was removed. 100 µL of fluorescent secondary antibody diluted to 1 $\mu g.mL^{-1}$ in 1% (w/v) BSA in PBS-T was added to each well and incubated for 1 hour at room temperature. The secondary antibody was Goat anti-Mouse Alexa 488. Following the removal of the secondary antibody the washing step was repeated as above. 100 µL of 1 $\mu g.mL^{-1}$, 4',6-diamidino-2-phenylindole (DAPI) in 1% (w/v) BSA in PBS-T was added to each well and incubated for 15 mins a room temperature. The washing step was repeated as above, to remove excess DAPI. 50 µL of PBS-Azide was added to each well to prevent the drying out and to aid in visualisation.

#### 3.3.2.4.5 Cell imaging

Wells were viewed under a fluorescent microscope (Olympus IX70) using excitation wavelengths set at 360 nm and 485 nm to detect DAPI and Alexa 488 respectively. Images representative of the cells in each well were captured using a Hamamatsu C4247-95 ORCA 100 series camera and analysed with HCImage. HeLa cell nuclei fluoresced blue due to the DAPI stain whilst the bacteria fluoresced green.

#### 3.3.2.4.6 Dot blot

Transformed *E. coli* strains which displayed an increasing in binding to HeLa CC1 cells were grown overnight and lysed. The samples were standardized to $A_{280}$ OD 0.5 and 50 µL of each was transferred to nitrocellulose membrane via direct pipetting and vacuum drying. Using 3% (w/v) BSA (diluted in PBS-T) the non-specific binding sites were blocked for 1 hour at room temperature. After initial blocking CC1-Fc was diluted to 0.2 $\mu g \cdot mL^{-1}$ in 1% (w/v) BSA-PBST for 1 hour at room temperature. The membrane was then washed three times with PBS-T, the third wash was incubated for 5 min for before being decanted. 1 $\mu g.mL^{-1}$ Rabbit-anti-Human-IgG(Fc)-AP 1% (w/v) BSA in PBS-T was added to each strip and incubated for 1 hour at room temperature. The blots were washed as before and developed for 15 mins using developing buffer (6.6 $\mu L \cdot mL^{-1}$ of NBT and 3.3 $\mu L \cdot mL^{-1}$ of BCIP in AP buffer).

### 3.3.3 Transposon library

#### 3.3.3.1 Preparing electrocompetent cells

In order to induce uptake of the EZ-Tn5™ <KAN-2>Tnp Transposome™ via electroporation it was necessary to prepare electrocompetent *Moraxella catarrhalis* cells (Holm, Vanlerberg, Sledjeski and Lafontaine, 2003). A falcon tube containing 5 mL HBHI broth was inoculated with MX13L from a fresh HBHI plate and placed in a 250 RPM shaking incubator for 12 hours at 37 °C. the next day 1ml of liquid culture was reinoculated into 50 mL of HBHI broth and grown in a shaking incubator under the same conditions as before. Once the culture reached an $OD_{600}$ 0.6 it was placed on ice for 15 mins, from this point onwards the culture was kept ice cold. The culture was then divided into 4 sterile, chilled falcon tubes and centrifuged for 10 mins at 5000 RPM. The supernatant was decanted, and the pellet resuspended in ice cold 10% glycerol bringing the volume up to 50 mL before being spun as previously. This step of spinning and resuspension with glycerol was performed a further 3 times. The final resuspension of cells was performed using the residual glycerol after the supernatant was discarded (approximately 2 mL). 50 µL of the now electrocompetent cells were used for immediate electroporation whilst the rest were reserved in 100 µL aliquots and stored at -80 °C.

#### 3.3.3.2 Electroporation of *Moraxella catarrhalis* with transposon

Prior to electroporation a reaction mixture consisting of 50 µL aliquot of electrocompetent cells along with 1 µL of the EZ-Tn5™ <KAN-2>Tnp Transposome™ and 2 µL of nuclease free water provided with the transposon kit was prepared. This

reaction mixture was incubated at room temperature for 30 mins before being transferred to a 0.2 cm electroporation cuvette. The electroporation cuvette was inserted into the Genepulser apparatus chamber between the contacts for the electrodes. The electrocompetent *Moraxella catarrhalis* cells were transformed with the transposon via a single pulse using the settings 2.5 kV, 25 μF, and 200 Ω (Nordström, Blom, Forsgren and Riesbeck, 2004). The cuvette was immediately removed from the apparatus and 947 μL of SOC medium was added to the cuvette, before the total volume was transferred to a microcentrifuge tube and incubated for 1 hour at 37 °C to facilitate recovery and cell growth. After the recovery time had elapsed the 100 μL aliquots of the cell mixture were plated on 10 HBHI plates supplemented with 25 $\mu g \cdot mL^{-1}$ of kanamycin. The plates were grown overnight at 37 °C in a 5% $CO_2$ incubator.

#### 3.3.3.3 Transposon library screening

In order to analyse the CC1-FC binding capabilities of the *Moraxella catarrhalis* transformed with the transposon, a selection of individual colonies was picked from the surface of the HBHI Kan supplemented plates, using sterile toothpicks. The point of a sterile toothpick was used to transfer a single colony from the HBHI plate into the well of a 96 well tissue culture plate containing 300 μL of HBHI supplemented with 25 μg.mL-1 of Kan. In order to maximise the chances of identifying the target protein 5760 colony picks were performed for the whole transposon library six 96 well plates were filled per HBHI plate.

#### 3.3.3.3.1 Dot Blots

Colony picks were grown and 2 µL of each was transferred to nitrocellulose membrane via direct pipetting and air drying. A range of *Moraxella catarrhalis* strains were also grown and transferred using the same technique as controls. Once the dotted material had dried, 3% (w/v) bovine serum albumin (BSA; diluted in PBS-T) was used to block the non-specific binding sites for 1 hour at room temperature. After initial blocking the liquid was aspirated and 0.2 $\mu g.mL^{-1}$ of CC1 in 1% (w/v) BSA-PBST, was applied to the membrane for 1 hour at room temperature. The membrane was then washed three times with PBS-T, the third wash was incubated for 5 min for before being decanted. Anti-human-Fc conjugated to alkaline phosphatase diluted to 1 $\mu g.mL^{-1}$ in 1% (w/v) BSA in PBS-T with 0.05% (w/v) $NaN_3$ was added to each strip and incubated for 1 hour at room temperature. The blots were washed as before and developed for 15 mins using developing buffer (6.6 $\mu L \cdot mL^{-1}$ of NBT and 3.3 $\mu L \cdot mL^{-1}$ of BCIP in AP buffer).

Once the blots had developed any dots which showed weak/ zero binding to CC1 were then identify and those colonies selected for further study.

A series of successive immunodot blots were carried out to whittle down the target list, each blot followed much the same process. In each successive screen a more concentrated solution of CC1-Fc was utilised. 0.4 $\mu g.mL^{-1}$ of CC1-Fc in screen 2, 0.6 $\mu g.mL^{-1}$ of CC1-Fc in screen 3, 0.8 $\mu g.mL^{-1}$ of CC1-Fc in screen 4, 1 $\mu g.mL^{-1}$ of CC1-Fc in screen 5.

Colonies were grown and freeze thaw lysis was performed, lysates were then standardized to $OD_{260}$ 0.5 and 50 µL of each was transferred to nitrocellulose

membrane via direct pipetting and vacuum drying. Non-specific binding sites were blocked for 1hr at room temperature with 3% (w/v) bovine serum albumin (BSA; diluted in PBS-T). CC1-Fc diluted in 1% (w/v) BSA-PBST, was applied to the membrane for 1 hour at room temperature. The membrane was then washed three times with PBS-T, the third wash was incubated for 5 min. Anti-human-Fc conjugated to alkaline phosphatase diluted to 1 $\mu g.mL^{-1}$ in 1% (w/v) BSA in PBS-T with 0.05% (w/v) $NaN_3$ was added to each strip and incubated for 1 hour at room temperature. The blots were washed as before and developed for 15 mins using developing buffer (6.6 $\mu L \cdot mL^{-1}$ of NBT and 3.3 $\mu L \cdot mL^{-1}$ of BCIP in AP buffer).

#### 3.3.3.3.2 PCR

Colonies that did not bind to CC1, were subject to PCR with the BOMP specific primers. 7µL nuclease free water ($ddH_2O$), 0.5 µM of forward and reverse primers, as well as 1 $ng.\mu L^{-1}$ genomic DNA were added to 10 µL CloneAmp™ high fidelity PCR premix in a 0.2 mL PCR tube to form the PCR mix.

PCR was performed under the following conditions: initial denaturing 98 °C 5 min; followed by 30 cycles of: denaturing 98 °C 10 sec, annealing X °C 15 sec, extension 72 °C 5 $sec \cdot kBase^{-1}$ DNA; finished by 72 °C 5 min for the final extension. The annealing temperature of the primers varied (table 1).

Colonies in which PCR products which had increased by 1221 bp due to the insertion of the transposon were selected for sanger sequencing.

# 4 RESULTS – STABLE CC1 SECRETING CELL LINE

## 4.1 Creating a stable CC1-Fc secreting cell line

To examine the CC1 binding capabilities of *Moraxella catarrhalis* a large amount of CC1 was required on hand for multiple experiments. Prior to this project the laboratory had previously either bought CC1-Fc commercially or produced it transiently, both of these systems limited the amount of CC1 that could be accessed immediately. It was decided that in order to have as much CC1 as required, a stable CC1-Fc secreting cell line should be constructed.

## 4.2 Optimising the zeocin concentration required to select for successful transfection

Prior to any transfections taking place it was necessary to optimize the concentration of zeocin which would be used as a selective pressure, ensuring only successfully transfected cells survived. The zeocin resistance of the transfected cells was conferred by the *Sh ble* gene encoded by the pINFUSE-hIgG2-Fc2 plasmid. In order to determine the optimal zeocin concentration a kill curve was undertaken. This kill curve exposed untransfected CHO cells to a range of zeocin concentrations for a period of 10 days. Cells were checked daily under a microscope and a visual assessment of percentage survival was determined and antibiotic concentration was maintained. after a 10-day observation period the optimal zeocin concentration required for the selection of transfected CHO pINFUSE CC1-Fc cells was determined to be 400 $\mu g \cdot mL^{-1}$.

## 4.3 Amplifying the *CC1-Fc* gene from the full length *CC1* gene

In order to create a stable CC1 secreting cell line, it was necessary to first amplify and clone the CC1-Fc isoform encoding DNA. PCR was used to amplify the correct CC1 isoform from genomic DNA obtained from CC1 expressing HeLa cells (figure 5). Prior experiments examining CC1 had used primers which were unsuitable for amplifying CC1-Fc. As evident by the four bands in lane 3 & 4, these primers appeared to be binding to multiple sites which in turn produced undesirable non-specific products. As such new forward and reverse primers were designed which enabled the amplification of the transmembrane domain lacking isoform of CC1 only. This resulted in the 1000 kb band in lane 2 & 5 which, according to sequence data obtained from Uniprot, corresponded to the expected molecular weight of the target isoform. These primers were also designed to be used in ligation independent cloning and as such had 3' regions which were homologous with pINFUSE that had been linearized using *EcoR*V and *BglII* restriction enzymes. As both enzymes were compatible with NEBuffer 3.1 a double digest reaction was undertaken at 37 °C for 15 minutes which resulted in the complete linearization of the 4397 bp pINFUSE plasmid (figure 6).

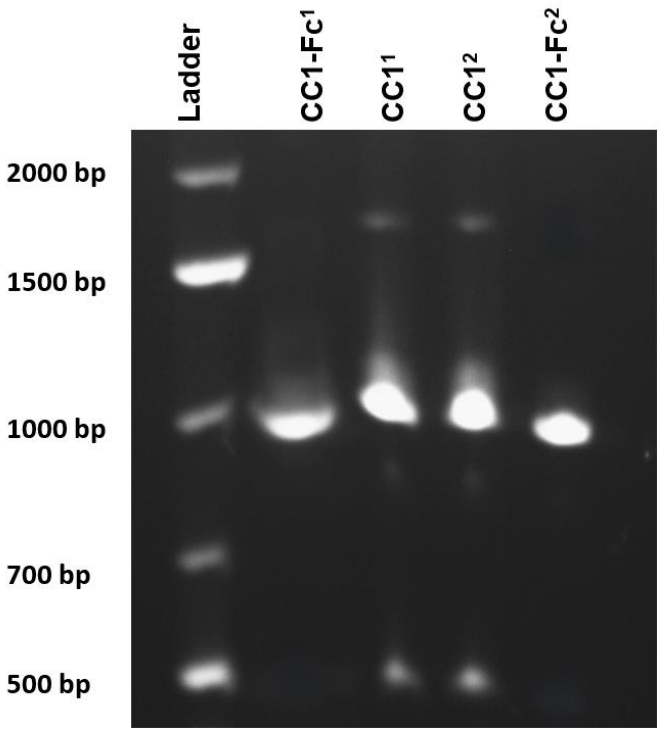



Figure 5 Amplification of human CC1-Fc from HeLa CC1 genomic DNA.

PCR of CC1-Fc using genomic DNA from HeLa CC1 cells using either the CC1 forward and reverse primers or CC1-Fc forward and reverse primers (appendix 1).

PCR product were separated using 0.8% agarose TBE gel electrophoresis. Lane 1 Generuler 1 kb Plus DNA ladder. Marker sizers are indicated in base pairs (bp).

Although PCR performed utilising older CC1 primers produced a band at approximately 1000 bp corresponding to the expected size of CC1-Fc isoform (Lane 3 & 4), multiple other products were also observed at 1500 bp, 900 bp, and 500 bp. New CC1-Fc specific primers were designed and produced a single product of approximately 1000 bp in size which corresponds with the expected size of the CC1-Fc isoform lacking any intracellular or transmembrane regions (Lane 2 & 5).

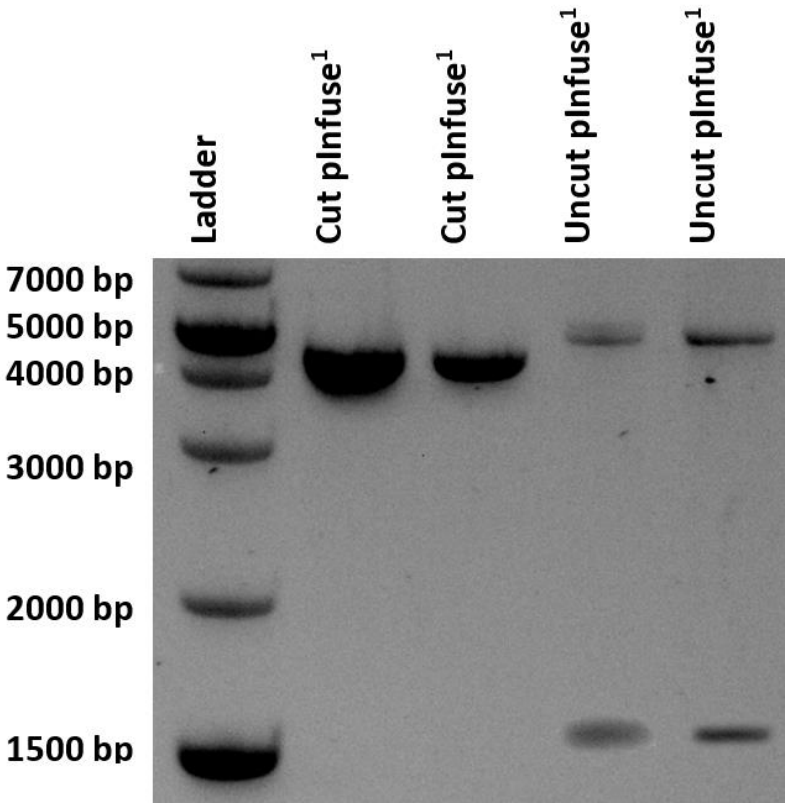



Figure 6 Linearization of pINFUSE-hIgG2-Fc2

Comparing supercoiled pINFUSE-hIgG2-Fc2 with the same plasmid linearized using the restriction enzymes EcoRV and BglII.

Plasmid DNA was separated using 0.8% agarose TBE gel electrophoresis. Lane 1 Generuler 1 kb Plus DNA ladder. Marker sizers are indicated in base pairs (bp).

Uncut plasmid (lane 4 & 5) produced two distinct products at approximately 5000 bp and 1500 bp which corresponds to 2 possible plasmid conformations. The larger band representing a relaxed confirmation whilst the smaller band represented a lower energy supercoiled state. When pINFUSE was digested with both EcoRV and BglII a product of approximately 4500 bp was produced (Lane 2 & 3), this corresponded with the actual size of the plasmid which was 4397 bp.

### 4.3.1 Cloning of CC1-Fc PCR product into XL10 Gold cells

Prior to transfection it was necessary to increase the amount of pINFUSE-CC1-Fc available and as the gene was to be transfected into eukaryotic cells, XL10 gold cells were used. Xl10 gold ultracompetent cells underwent heat shock in the presence of the plasmid and were plated onto LB plates supplemented with zeocin for overnight growth. After a period of overnight growth over 100 distinct colonies were observed whilst the control plate using untransformed showed no growth, ten colonies were selected and expanded in zeocin supplemented LB broth. Two of the ten colonies did not grow when placed in the growth medium. The remaining eight colonies were subject to plasmid extraction via the QAIGEN MiniPrep kit; during this process four of the colonies were mishandled and thus excluded from the rest of the screening process. The four remaining colonies had the plasmid successfully extracted and were examined using PCR and gel electrophoresis (figure 7) which confirmed that transformation was successful in all four of the chosen colonies.

After the successful transformation and extraction of pINFUSE-CC1-Fc from XL10-Gold cells, the plasmid was relinearized (figure 8). The NotI restriction enzyme was used to create a linear pINFUSE plasmid containing CC1-Fc in order to increase the chances of integration with host cell genomes thus enabling continuous stable protein expression. The single 5398 bp band in lane 1 corresponded to the expected size of completely linear pINFUSE-CC1-Fc.

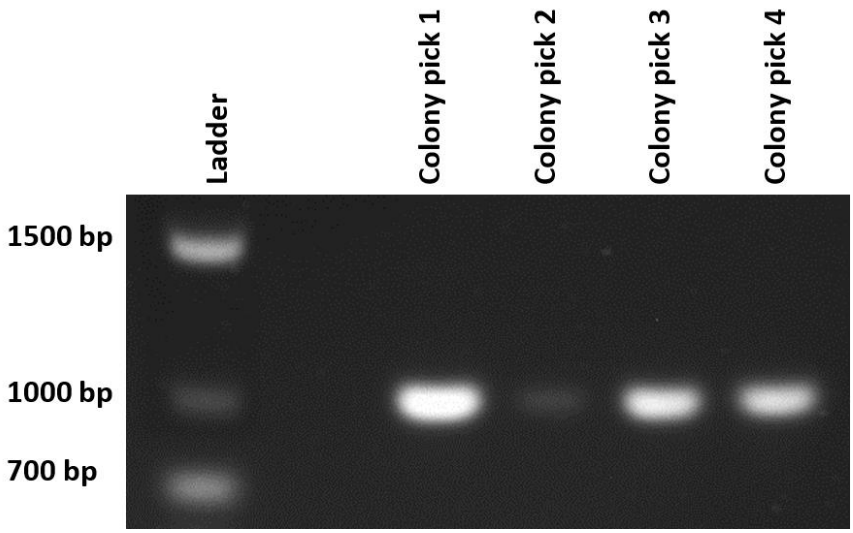



Figure 7 Amplification of CC1-Fc from transformed XL10 gold cells

PCR of CC1-Fc using plasmid DNA extracted from XL10 gold cells transformed with pINFUSE-CC1-Fc using the CC1-Fc forward and reverse primers (appendix 1). PCR product were separated using 0.8% agarose TBE gel electrophoresis. Lane 1 Generuler 1 kb Plus DNA ladder. Marker sizers are indicated in base pairs (bp).

Colony picks 1, 3, and 4 all produced a single product of approximately 1000 bp in size which corresponds with the expected size of the CC1-Fc isoform lacking any intracellular or transmembrane regions (Lanes 3, 5, 6). Colony pick 2 also produced a single product at approximately 1000 bp although it was much fainter than the bands produced by the other colony picks (lane 4).

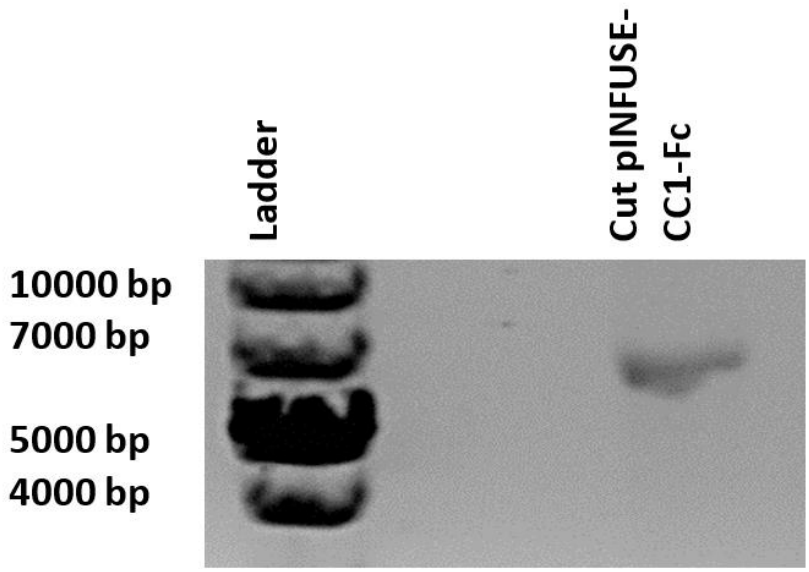



Figure 8 Linearization of pINFUSE-CC1-Fc

Linearisation of the pINFUSE-CC1-Fc plasmid with the NotI restriction enzyme in preparation for transfection.

Plasmid DNA was separated using 0.8% agarose TBE gel electrophoresis. Lane 1 Generuler 1 kb Plus DNA ladder. Marker sizers are indicated in base pairs (bp).

When pINFUSE-CC1-Fc was digested with NotI it produced a single linear product of approximately 5500 bp (Lane 3), this corresponded with the expected combined size of the plasmid and the gene of interest.

Data representative of products obtained on several occasions; repeats performed by undergraduate student under my supervision.

## 4.4 Assessing for CC1 production in transfected cells.

CHO cells were transfected with linear pINFUSE-CC1-Fc DNA using the lipid-based transfection reagent lipofectamine 3000. Prior to selection of a monoclonal population it was necessary to determine that the transfected cells were producing CC1, as such the supernatant from the transfected cells was collected.

The supernatant from the transfected CHO cells was subject to serial dilutions (figure 9), dots 1 through 3 demonstrated a binding for A0115 that was significantly higher than background. Whilst dot four displayed reduced binding equal to levels observed in the negative control dots. The last dot in the series displayed no binding for the anti-CEACAM antibody at all, below the background level binding displayed in the negative control dots. Although it is not clear why the binding of dot 5 was below that of supernatant from untransfected cells, it was clear that the first three dots were binding to A0115 significantly stronger than the controls.

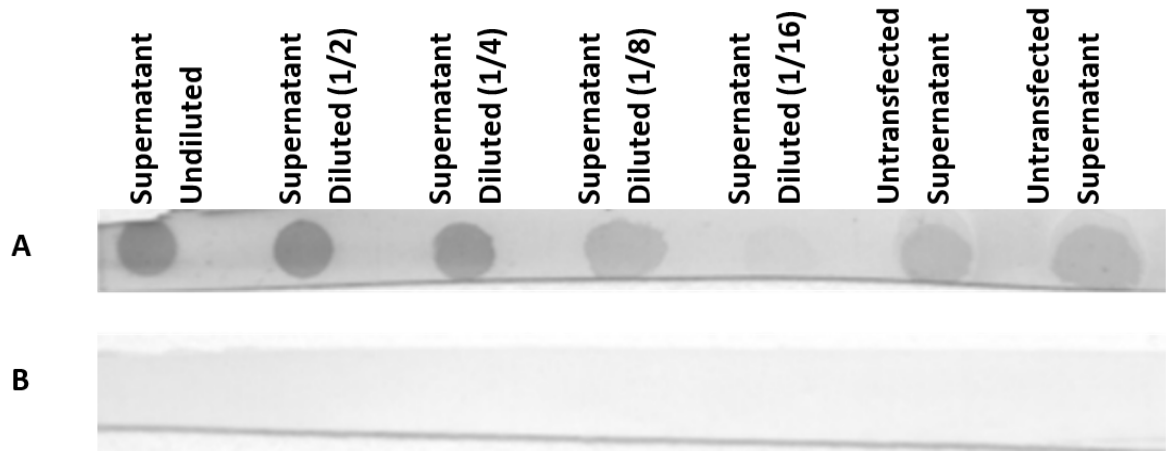



Figure 9 Assessing the CC1-Fc secretion of transfected CHO cells.

A dot blot was performed to determine if the transfected CHO cells were secreting CC1-Fc. Supernatant was collected from the CHO cells transfected with the pINFUSE-CC1-Fc DNA at days 3 and 7 post transfection. The collected supernatant was pooled, and a series of serial dilutions were carried out were each successive dot was half as dilute as the previous dot (dot 1-5). Supernatant obtained at the same intervals from untransfected CHO cells were used as negative controls (dots 6-7).

Membrane A was overlaid with primary antibody 1 $\mu g.mL^{-1}$ A0115, then secondary antibody 1 $\mu g.mL^{-1}$ Goat-anti-Rabbit-IgG(Fc)-AP.

Transfected supernatant dilutions 1-3 produced dots which were significantly darker than the negative control dots indicating that CC1 was detected in the supernatant. Dilution 4 produced a dot which was comparable to the control dots, suggesting that at this level of dilution no CC1 could be detected. Dilution 5 produced a dot that was weaker than the negative control dots.

Membrane B acted as a negative control with only a secondary antibody overlay. Data representative of products obtained on several occasions; repeats performed by undergraduate student under my supervision.

## 4.5 Assessing the production of CC1-Fc by monoclonal populations of transfected CHO cells.

After confirmation that the transfected CHO cells were producing CC1-Fc, some of the cells were reseeded into a 96 well plate in order to select for a monoclonal CC1 secreting population, whilst the rest were reserved as a polyclonal population in a T75 flask. After a period of selection 5 monoclonal populations were identified and their growth expanded in T25 flasks. During passaging the supernatant of all monoclonal populations was collected and examined via immune dot blot.

50 µL of each monoclonal supernatant was vacuum dotted onto nitrocellulose membrane and overlaid with A0115 (figure 10) in order to assess the comparative amounts of CC1-Fc produced by each of the monoclonal populations. The supernatant from each of the monoclonal populations visually demonstrated a significantly higher binding to A0115 compared to the control blots containing the supernatant from untransfected cells. No single monoclonal population bound more strongly to A0115 than the other which indicated that each population was producing a similar amount of CC1-Fc.

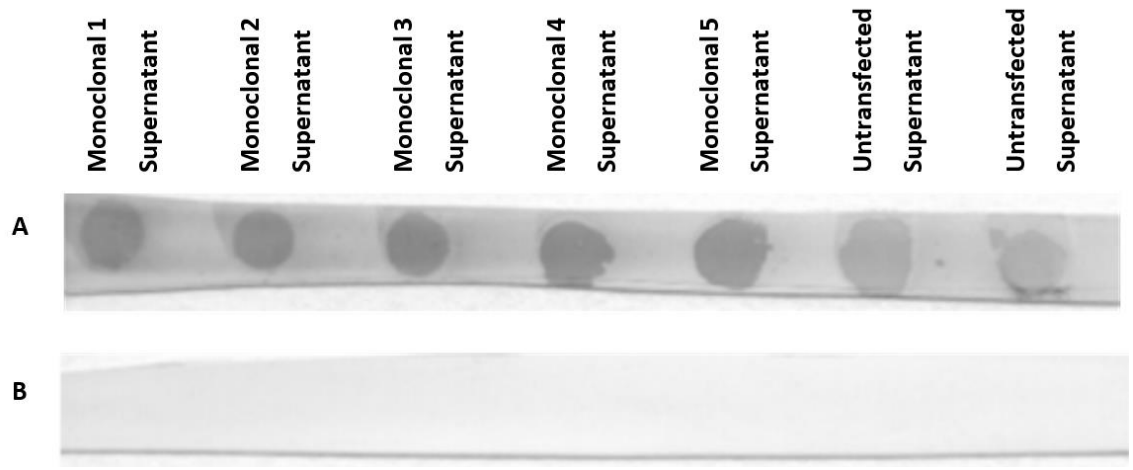



Figure 10 Assessing the CC1-Fc production of monoclonal CC1-Fc secreting CHO populations

A dot blot was performed to determine if the monoclonal populations of transfected CHO cells were secreting CC1-Fc. Supernatant was collected from the selected monoclonal populations of CHO cells transfected with the pINFUSE-CC1-Fc DNA. The collected supernatants were blotted onto nitrocellulose membrane (dots 1-5) Supernatant obtained from untransfected CHO cells were used as negative controls (dots 6-7).

Membrane A was overlaid with primary antibody 1 $\mu g.mL^{-1}$ A0115, then secondary antibody 1 $\mu g.mL^{-1}$ Goat-anti-Rabbit-IgG(Fc)-AP. All transfected supernatants produced dots which were visually stronger than the background levels observed in the negative control dots indicating that CC1-Fc was secreted by all monoclonal populations.

Membrane B acted as a negative control with only a secondary antibody overlay.

Data representative of products obtained on several occasions; repeats performed by undergraduate student under my supervision.

## 4.6 Determining the concentration of CC1-Fc

Before CC1-Fc could be of any real use in experiments it was necessary to determine the concentration of the purified protein. This was done via BCA assay. This assay was selected as it has a high sensitivity over a wide range of concentrations, enabling precise results as long as the protein concentration is between the ranges 20 $\mu g \cdot mL^{-1}$ or 2000 $\mu g \cdot mL^{-1}$. The BCA assay determined the CC1-Fc concentration to be 227 $\mu g \cdot mL^{-1}$ (figure 11).

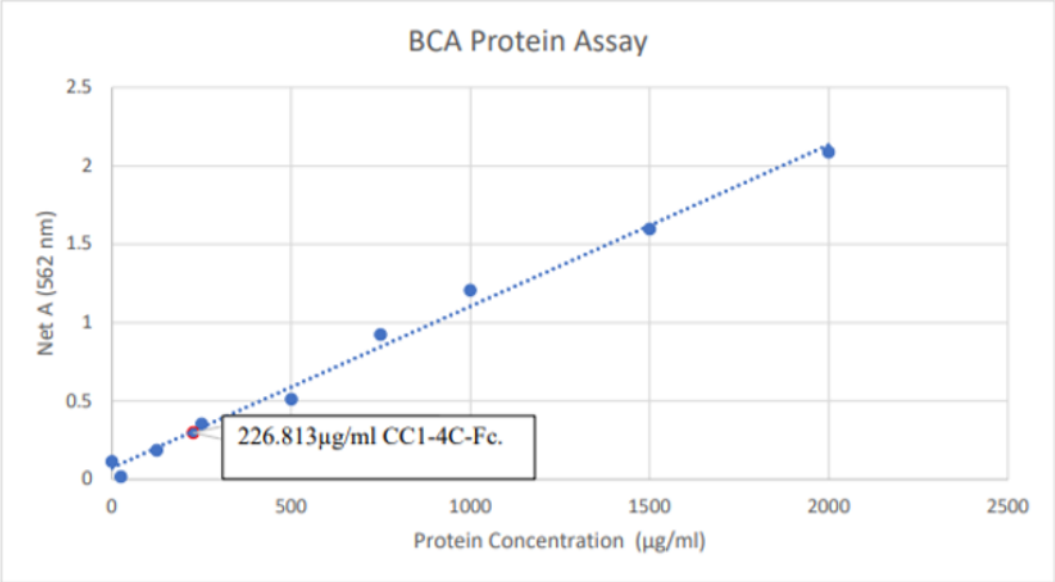



Figure 11 Determining the concentration of purified CC1-Fc

A BCA assay was performed to assess the concentration of the CC1-Fc secreted by the transfected CHO cells. After purification via protein Sepharose A, the CC1-Fc was compared to a set of standards ranging from 20-2000 µg.ml-1. The concentration of the protein was proportional to the amount of reduced $Cu^{2+}$, which corresponds to the intensity of the resulting purple coloured complex. A reading was taken at 562 nm (the wavelength most strongly absorbed by the 2BCA-$Cu^{+}$ complex) and a graph plotted. The concentration of CC1-Fc (indicated by the red dot) was determined in comparison to standards.

## 4.7 Comparing the rD-7 binding of CC1-Fc and oCC1-Fc.

To examine the fidelity of the newly produced CC1-Fc another immune dot bot was undertaken which compared its binding capabilities to a verified CC1-Fc sample. An OpaLess strain of Neisseria and rD-7, a recombinant peptide spanning the CEACAM binding region of UspA1 were vacuum blotted onto nitrocellulose membrane in triplicate. The non-specific binding sites were blocked before being overlaid with either CC1-Fc produced in the laboratory or the oCC1-Fc (figure 12). The strong binding to rD-7 appeared to be identical for both CC1-Fc and oCC1-Fc. Whilst no binding occurred between either of the CC1-Fc samples and the OpaLess negative control, as expected.

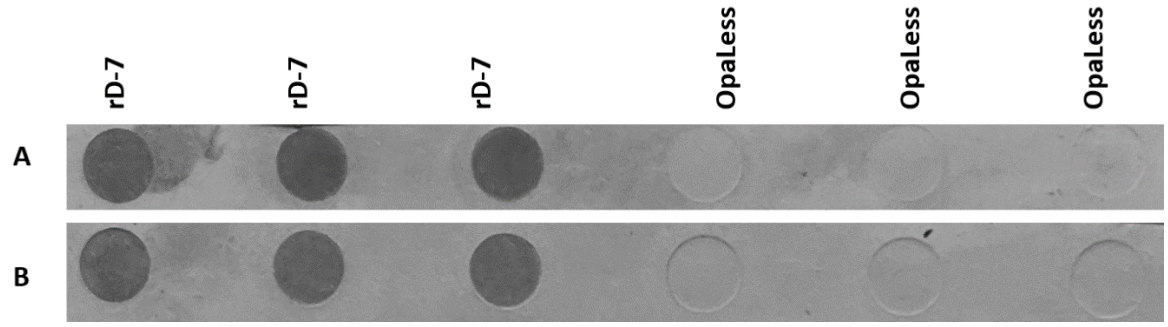



Figure 12 Comparing the binding of CC1-Fc and oCC1-Fc

A dot blot was performed to determine if the secreted CC1-Fc produced by the transfected CHO cells performed the same as oCC1-Fc when used experimentally. Purified rD-7 (dots 1-3) and OpaLess (dots 4-6) were blotted onto nitrocellulose membranes.

Membrane A was overlaid with CC1-Fc 0.2 $\mu g.mL^{-1}$, then secondary antibody 1 $\mu g.mL^{-1}$ Rabbit-anti-Human-IgG(Fc)-AP.

Membrane B was overlaid with oCC1-Fc 0.2 $\mu g.mL^{-1}$ then secondary antibody 1 $\mu g.mL^{-1}$ Rabbit-anti-Human-IgG(Fc)-AP.

Both membrane A and B developed identically, as expected dots 1-3 displayed strong binding to both CC1-Fc and oCC1-Fc, whilst dots 4-6 did not bind to either CC1-Fc or oCC1-Fc.

Data are representative of products obtained on several occasions.

## 4.8 SDS-Page gel and western blot to determine the purity of CC1-Fc.

Whilst immune dot blots showed that the CC1-Fc bound to rD-7 and OpaLess identically to oCC1-Fc it was necessary to verify that the proteins also behaved the same under different conditions. An SDS-Page gel was performed to compare the purity and the molecular weights of both CC1-Fc and oCC1-Fc as well as older stock sample of CC1 (figure 13). Although CC1-FC is a is predicted to be a 57.7 kDa protein it is highly glycosylated and often dimerises therefore appearing much larger on SDS-Page gels than in actuality. Both CC1-Fc (lane 1) and oCC1-Fc (lane 2 & 3) show faint bands at approximately 57 kDa as well as stronger bands at 250 kDa. The older CC1 sample in lane 5 shows a strong band at 50 kDa, which could be an antibody fragment, another band may also have been seen at approximately 25 kDa but it was run off the gel. The sample in lane 5 also demonstrated extensive laddering, this is indicative of a contaminated or degraded sample, possibly due to fragmentation or overuse of the sample.

A western blot was performed to compare the functions of CC1-Fc to oCC1-Fc under denaturing conditions. The proteins were run on an SDS-PAGE gel before being transferred to nitrocellulose and overlaid with the CEACAM specific antibody A0115 (figure 14), which binds to the N terminus of the protein. Both CC1-Fc (lane 2) and oCC1-Fc (lane 3 & 4) show strong diffuse bands at 250 kDa, although the band in lane 3 was stronger than those of either lane 2 or 4.

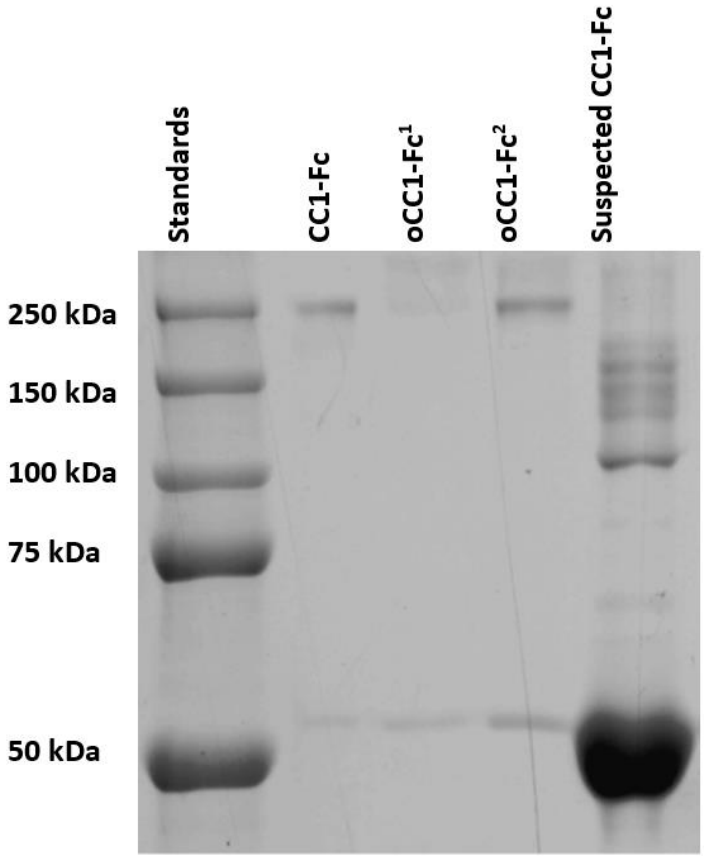



Figure 13 SDS-Page gel comparing CC1-Fc to oCC1-Fc

An SDS-Page gel was run and developed via Coomassie stain, to visually compare the molecular weights of CC1-Fc to two fractions of oCC1-Fc, as well as an older stock that was suspected to be CC1.

Protein samples were separated by running a 7.5% resolving gel and 4% stacking gel at 200 V for 1 hour 30 minutes. Lane 1 Precision Plus Protein Dual Colour Standards. Marker sizers are indicated in kilo-Daltons (kDa).

Strong bands at approximately 250 kDa were evident in all four samples of protein, this is significantly larger than the expected weight of CC1-Fc as indicated by its protein sequence but does correspond to the expected size of the protein when the extensive glycosylation of CC1 is considered. Faint bands at approximately 50 kDa were also present in lanes 2-4 (CC1-Fc, oCC1-Fc1, and oCC1-Fc2), these bands could be antibody fragments. This 50 kDa band was also present in lane 5 (suspected CC1 stock) although it was significantly stronger than the bands from the other samples. In contrast to lanes 2-4, the suspected CC1 stock (lane 5) also displayed multiple other bands between 250 kDa and 37 kDa in size, which indicated that this sample was impure.

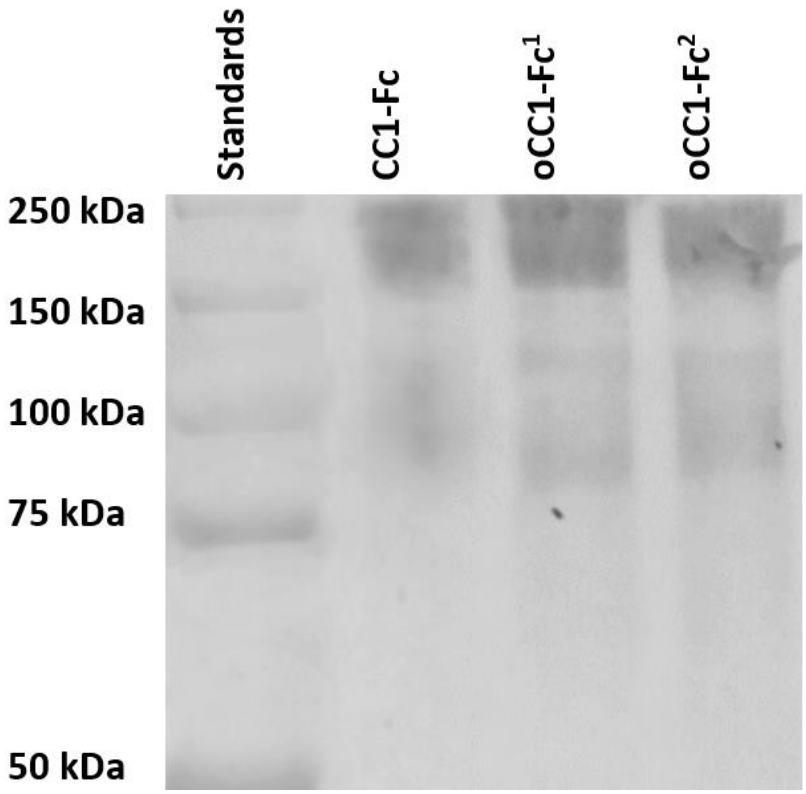



Figure 14 Western blot comparing rCC1 to vCC1.

A western blot was performed, to visually compare the fidelity of CC1-Fc to two fractions of oCC1-Fc. Proteins were separated by SDS-Page gel and the gel transferred to nitrocellulose membrane at 350 mA for 60 min. Once transferred the membrane was overlaid with primary antibody A0115 and secondary antibody Goat-anti-Rabbit-IgG(Fc)-AP both at a concentration of 1 µg.ml-1. Lane 1 Precision Plus Protein Dual Colour Standards. Marker sizers are indicated in kilo-Daltons (kDa).

Strong diffuse bands were present at approximately 250 kDa were evident in lanes 2-4 (CC1-Fc, oCC1-Fc1and oCC1-Fc2), which corresponds to the expected size of CC1-Fc as determined in figure 13. The band in lane 3 was stronger than the band produced by CC1-Fc (lane 2) this may be due to disparities in concentration of the tested proteins.

Data representative of products obtained on several occasions; repeats performed by undergraduate student under my supervision.

## 4.9 Conclusions

Overall the results of the experiments suggest that *CC1-Fc* gene was successfully transfected into the CHO cell genome, resulting in a stable CC1-Fc secreting cell line. The purified protein responds functionally identically to stock CC1 in interactions with both A0115 and rD-7. The total concentration of CC1-Fc purified from 1.5 L of supernatant was 226.8 $\mu g \cdot mL^{-1}$, the supernatant in this case was obtained from both poly and monoclonal populations of transfected cells. In the future purifying CC1-Fc from the supernatant of monoclonal clonal populations may produce a higher concentration of CC1-Fc.

# 5 RESULTS – ASSESSING THE CC1 BINDING PROPERTIES OF MX13L

## 5.1 CC1 adhesive properties of *Moraxella catarrhalis* strains

Unpublished observations within the Hill group identify two variants of ATCC25240 with different CC1 binding properties, termed MX13L and MX13D. Although ATCC25240 expressed a truncated UspA1 protein lacking the rD-7 region, the MX13L variant was shown to bind to CC1, despite lacking the functional region required to do so. A number of experiments where undertaken to confirm that MX13L did in fact lack an rD-7 region and still bind to CC1.

### 5.1.1 Assessing the binding of MX13L to CC1

*Moraxella catarrhalis* strains MX13L, MX13D, ATCC25240, MX13ΔUspA1, MX2 and ATCC25239 were selected to compare their binding to CC1-Fc. Purified rD-7 acted as a positive control, whilst the OpaLess strain of *Neisseria* acted as a negative control. The selected strains were grown overnight and standardised to $A_{280}$ OD 0.5. The non-specific binding sites were blocked before being overlaid with either CC1-Fc or oCC1-Fc (figure 15). As expected, MX2 and rD-7 showed strong binding to CC1 this was due to the high specificity of the rD-7region for CC1. MX13L and MX13ΔUspA showed reduced receptor binding compared to rD-7. Whilst at a lower level than that observed for MX2 or pure rD-7, the CC1-Fc binding of both MX13L and MX13ΔUspA was greater than MX13D or the parent strain ATCC25240. Additional controls included an overlay with anti-Human only, as expected this negative control showed no binding by any of the isolates.

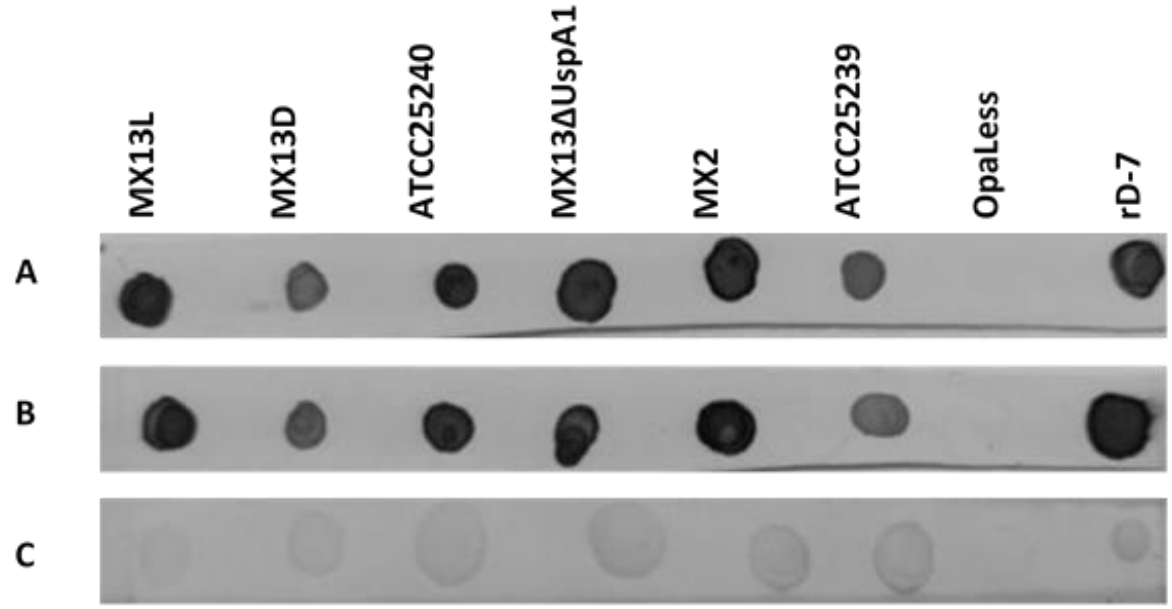



Figure 15 Dot blot assessing the CC1 binding of MX13L

A dot blot was performed assess the CC1 binding capabilities of various *Moraxella catarrhalis* strains. All lysates and proteins were standardised to OD600 0.5. The lysates of selected *Moraxella catarrhalis* strains; MX13L, MX13D, ATCC25240, MX13ΔUspA1, MX2, and ATCC25239 were blotted onto nitrocellulose membrane (dots 1-6). OpaLess (dot 7) and rD-7 (dot 8) acted as negative and positive controls respectively.

Membrane A was overlaid with primary antibody 1 $\mu g.mL^{-1}$ CC1-Fc. Membrane B was overlaid with 1 $\mu g.mL^{-1}$ oCC1-Fc. Both membranes A & B were overlaid with secondary antibody 1 $\mu g.mL^{-1}$ Rabbit-anti-Human-IgG(Fc)-AP.

Membrane C acted as a negative control and was only overlaid with secondary antibody.

As expected, dots 5 & 8 showed very strong binding. Dots 1 & 4 also displayed strong binding to CC1, despite both lacking the UspA1 protein. Dots 2, 3 & 6 all displayed reduced binding to CC1. All of the *Moraxella catarrhalis* strains displayed a binding for CC1 greater than OpaLess.

Data representative of products obtained on several occasions.

### 5.1.2 Comparing the UspA1 sequences of the MX13L parent strain ATCC25240 to a functional UspA1 protein

Pairwise alignment between a functional UspA1 sequence and the ATCC25240 sequence highlights the incomplete nature of the UspA1 protein produced by ATCC25240 (figure 16). There are extensive regions of the BBH18 UspA1 sequence that are not present in the ATCC25240 sequence as well as amino acid substitutions in the regions that do overlap. The analysis of both sequences also demonstrates that whilst the minimal cc1 binding region of rD-7 is clearly present in the BBH18 UspA1 protein no such sequence is evident in the ATCC25240 protein.

Figure 16 Amino acid sequence comparison of functional a UspA1 protein and the UspA1 protein of ATCC25240

Pairwise alignment of the UspA1 protein sequences of the *Moraxella catarrhalis* strains BBH18 and ATCC25240 was performed using the 'Multiple sequence alignment with hierarchical clustering' tool. Identical amino acids between each of the proteins are highlighted in blue whilst the predicted minimal CC1 binding sequence of the rD-7 region is shown in red.

### 5.1.3 Determining the presence of the UspA proteins

Pairwise alignment data was corroborated by the use of UspA1 and UspA2V specific primers, neither of which produced a band with ATCC25240 (figure 17). In the case of UspA2V this was because ATCC25240 does not encode a copy of this gene in its genome. Although ATCC25240 does encode a *UspA1* gene it is heavily truncated and missing the part of the sequence required for successful amplification with the UspA1 distinguishing primer.

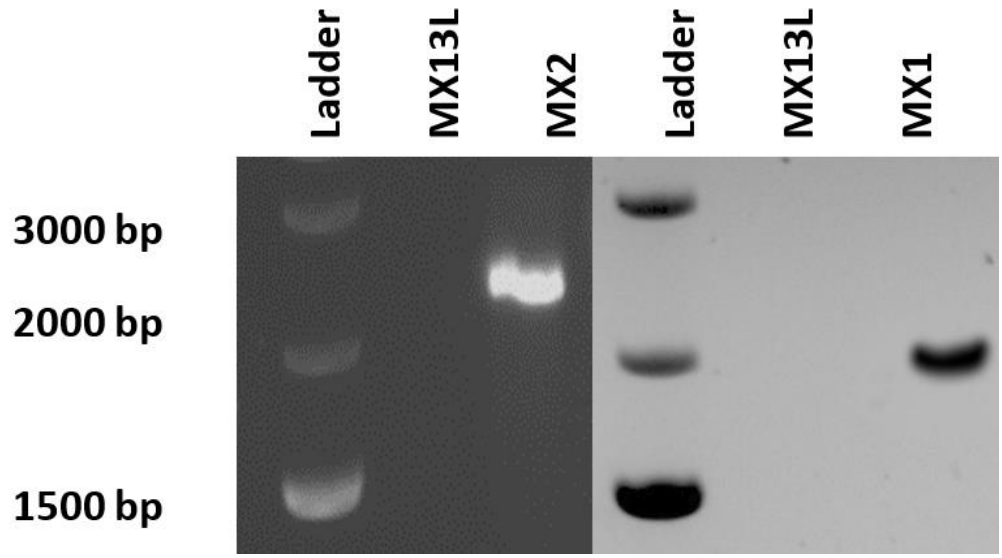



Figure 17 Amplification of UspA1 and UspA2 from MX13L

PCR of UspA1 and UspA2 from either MX13L, MX2 or MX1 DNA using either the UspA1 or UspA2 primers (appendix 1). PCR product were separated using 0.8% agarose TBE gel electrophoresis. Lane 1 & 4 Generuler 1 kb Plus DNA ladder. Marker sizers are indicated in base pairs (bp).

PCR performed utilising UspA1 primers in conjunction with MX13L DNA (lane 2) produced no band, in contrast PCR with the same primers against MX2 DNA (lane 3) produced a single band at approximately 2500 bp. Using the UspA2 primers to amplify MX13L DNA (lane 5) also produced no product, whilst amplification of MX1 DNA (lane 6) produced a single 2000 bp band. This indicated that MX13L did not encode for either UspA1 or UspA2. Due to sequence similarities between UspA2 and UspA2V & H, primers for UspA2 would also have amplified either of the variant genes as well (as in the case of MX1 which encodes UspA2V).

### 5.1.4 Determining the presence of the CC1 binding region rD-7

Selected *Moraxella catarrhalis* lysates were dotted onto a nitrocellulose membrane and overlaid with an anti-rD-7 antibody, R38 (figure 18). Purified rD-7 acted as a positive control, whilst a duplicate membrane overlaid with secondary antibody only acted as a negative control. As expected MX2 and rD-7 were bound by anti-rD-7. *Moraxella catarrhalis* strains, MX13L to MX13D and MX13ΔUspA2 displayed comparable binding to the negative control strip. All lysates produced a negative result when blocked with anti-Rabbit only (data not shown). This experiment in conjunction with previous observations, including a proteinase k digest, proved that MX13L was not expressing the traditional *Moraxella catarrhalis* CC1 binding protein and therefore another protein must be responsible for CC1 adhesion.

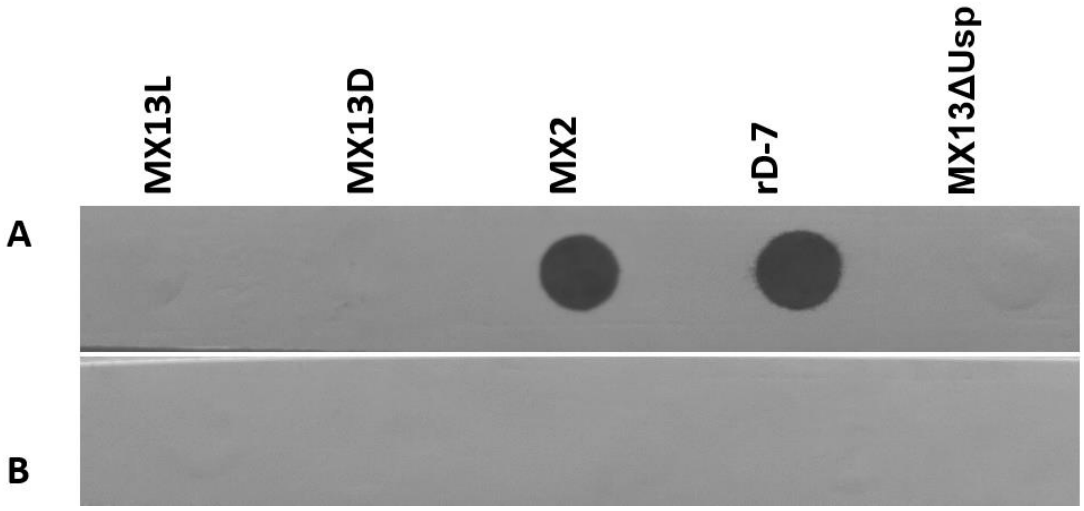



Figure 18 Dot blot to detect the rD-7 region.

A dot blot was performed to detect the presence of the rD-7 region in various *Moraxella catarrhalis* strains. All lysates and proteins were standardised to OD600 0.5. The lysates of selected *Moraxella catarrhalis* strains; MX13L, MX13D and MX2 were blotted onto nitrocellulose membrane (dots 1-3). rD-7 (dot 4) and MX13ΔUsp (dot 5) acted as negative and positive controls, respectively.

Membrane A was overlaid with primary antibody 1 $\mu g.mL^{-1}$ anti-rD-7, then secondary antibody 1 $\mu g.mL^{-1}$ Goat-anti-Rabbit-IgG(Fc)-AP.

Membrane B acted as a negative control with only a secondary antibody overlay.

Dots 1, 2 and 5 did not produce a dot and therefore did not bind to the anti-rD-7 antibody. Whilst as expected dots 3 and 4 both produced a strong reaction to the primary antibody. These results indicate that neither MX13L nor MX13D were producing a rD-7 region.

Data representative of products obtained on several occasions.

### 5.1.5 Looking for the CC1 binding ligand

Following the determination that the rD-7 region of UspA was not responsible for the CC1 binding properties of MX13L, a western blot was run to determine the molecular weight of the novel protein (figure 19). The samples were boiled for 5 mins to denature the proteins in the lysate. The SDS-Page gel was run at 200 V for 1.5 hrs and the proteins were then transferred to a nitrocellulose membrane. The membrane was then overlaid with 0.2 $\mu g \cdot mL^{-1}$ CC1-Fc. MX2 produced a band at 100 kDa which corresponds with UspA1. MX13L, MX13D and ATCC25240 as well as OpaLess all produced no bands. Although OpaLess was expected to produce no bands as it lacked the required adhesin, the other Mx isolates were expected to produce bands as they do bind CC1. The total lack of bands means that the novel CC1 adhesin is heat or detergent modifiable, which could be indicative of it being a more labile protein than UspA1 e.g. a beta barrel protein. Additional attempts to elucidate the molecular weight of the protein were unsuccessful, as native western blots produced a similar result as above. Unlike the CC1 specific binding sequence of UspA1, the highly labile nature of the binding protein suggests that binding is brought about by the confirmation of the protein.

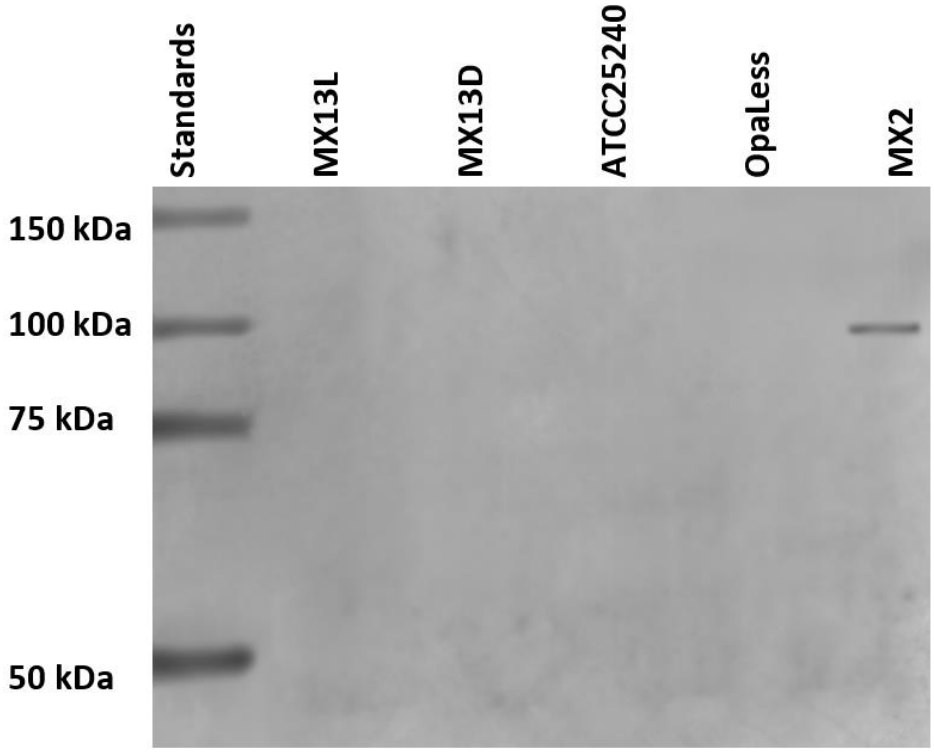



Figure 19 Western blot to compare the CC1 binding of various *Moraxella catarrhalis* strains

A western blot was performed to identify the molecular weight of the novel CC1 binding protein. Proteins were separated by SDS-Page gel and the gel transferred to nitrocellulose membrane at 350 mA for 60 min. Once transferred the membrane was overlaid with primary antibody CC1 and secondary antibody Rabbit-anti-Human-IgG(Fc)-AP both at a concentration of 1 $\mu g.mL^{-1}$. Lane 1 Precision Plus Protein Dual Colour Standards. Marker sizers are indicated in kilo-Daltons (kDa).

No bands were evident in lanes 2-4 (MX13L, MX13D, ATCC25240), which indicates that the novel adhesin is heat modifiable. Lanes 5 and 6 (OpaLess and MX2) acted as negative and positive controls respectively, with MX2 (lane 6) producing a single band at approximately 100 kDa.

Data representative of products obtained on several occasions.

## 5.2 Identifying a Novel CC1 binding protein

Experiments analysing the CC1 binding capabilities of *Moraxella catarrhalis* species MX13L identified a novel CC1 binding protein, separate from the traditional adhesin UspA1, which is highly labile. The heat sensitive nature of this protein indicates that it is potentially a beta barrel, as other labile adhesins such as the OPA proteins of *Neisseria* are also beta barrel structures. In order to create a potential target list of proteins a bioinformatic analysis of the proteins in ATCC25240 was required.

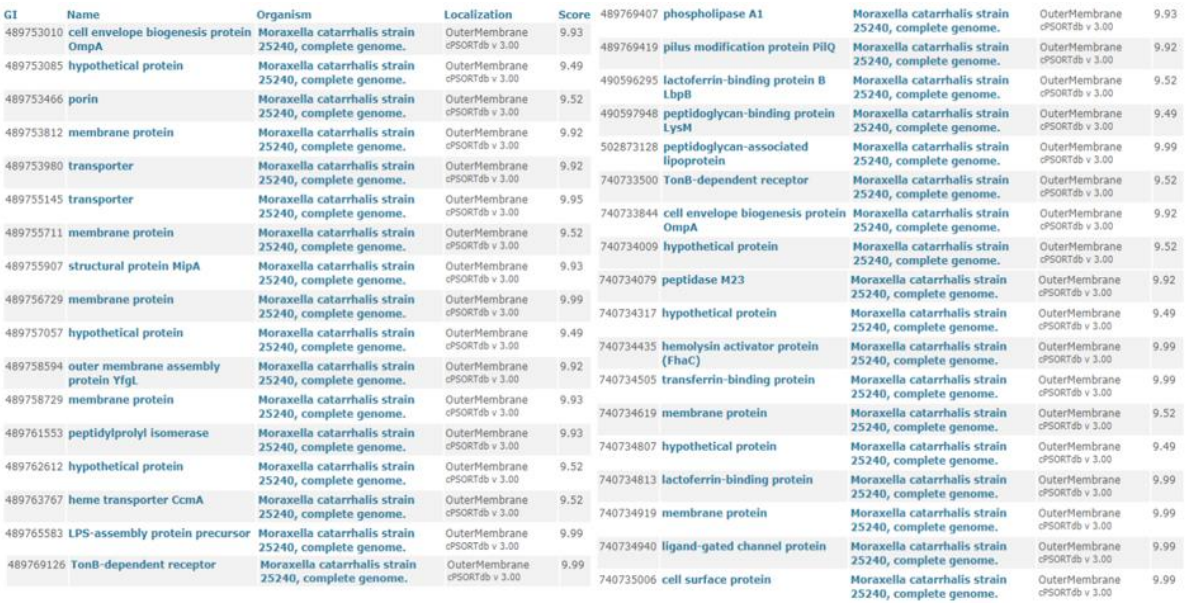
### 5.2.1 Narrowing down the list of target proteins

In order to narrow down the potential list of target proteins the PSORTdb was used to determine the subcellular localisation of all proteins found in the parent strain ATCC25240. PSORTdb is a database collating the subcellular localisation of proteins for bacteria and archaea. The information contained in the database is derived from laboratory verified experiments as well as computational predictions. PSORTdb generated a breakdown of the proteins present at each subcellular localisation. Of the 1687 proteins identified in ATCC25240 only 2% where predicted to be outer membrane proteins (figure 20). Unfortunately, PSORT cannot predict the structure of a protein. In order to identify which of the outer membrane proteins were beta barrel structures another program was used.

In order to further narrow down the list it was necessary to identify which of the outer membrane proteins were beta barrel structures, as such a program called BOMP was utilised. BOMP is a program which can predict if a protein is a beta barrel outer membrane protein from the protein's amino acid sequence. The β-barrel Outer

Membrane protein Predictor (BOMP) program identifies BOMPs through a two-part system. First it analyses the given sequence for the specific C-terminal pattern typical of many integral β-barrel proteins. The second step the program undertakes is searching the sequence for stretches of amino acids typical of transmembrane β-strands. Using BOMP reduced the number of potential targets from 35 to 19.

After analysing the genome of ATCC25240 with PSORT and BOMP, a list of 19 potential targets was generated. Using the NCIB accession sequence assigned to each of the proteins, the nucleotide sequence of each protein was obtained. Accessing the accession sequence of each protein identified 2 proteins which were duplicates, as such the target list was further reduced to 17 proteins (Table 2).



| GI | Name | Organism | Localization | Score |
|---|---|---|---|---|
| 489753010 | cell envelope biogenesis protein OmpA | Moraxella catarrhalis strain 25240, complete genome. | OuterMembrane cPSORTdb v 3.00 | 9.93 |
| 489753085 | hypothetical protein | Moraxella catarrhalis strain 25240, complete genome. | OuterMembrane cPSORTdb v 3.00 | 9.49 |
| 489753466 | porin | Moraxella catarrhalis strain 25240, complete genome. | OuterMembrane cPSORTdb v 3.00 | 9.52 |
| 489753812 | membrane protein | Moraxella catarrhalis strain 25240, complete genome. | OuterMembrane cPSORTdb v 3.00 | 9.92 |
| 489753980 | transporter | Moraxella catarrhalis strain 25240, complete genome. | OuterMembrane cPSORTdb v 3.00 | 9.92 |
| 489755145 | transporter | Moraxella catarrhalis strain 25240, complete genome. | OuterMembrane cPSORTdb v 3.00 | 9.95 |
| 489755711 | membrane protein | Moraxella catarrhalis strain 25240, complete genome. | OuterMembrane cPSORTdb v 3.00 | 9.52 |
| 489755907 | structural protein MipA | Moraxella catarrhalis strain 25240, complete genome. | OuterMembrane cPSORTdb v 3.00 | 9.93 |
| 489756729 | membrane protein | Moraxella catarrhalis strain 25240, complete genome. | OuterMembrane cPSORTdb v 3.00 | 9.99 |
| 489757057 | hypothetical protein | Moraxella catarrhalis strain 25240, complete genome. | OuterMembrane cPSORTdb v 3.00 | 9.49 |
| 489758594 | outer membrane assembly protein YfgL | Moraxella catarrhalis strain 25240, complete genome. | OuterMembrane cPSORTdb v 3.00 | 9.92 |
| 489758729 | membrane protein | Moraxella catarrhalis strain 25240, complete genome. | OuterMembrane cPSORTdb v 3.00 | 9.93 |
| 489761553 | peptidylprolyl isomerase | Moraxella catarrhalis strain 25240, complete genome. | OuterMembrane cPSORTdb v 3.00 | 9.93 |
| 489762612 | hypothetical protein | Moraxella catarrhalis strain 25240, complete genome. | OuterMembrane cPSORTdb v 3.00 | 9.52 |
| 489763767 | heme transporter CcmA | Moraxella catarrhalis strain 25240, complete genome. | OuterMembrane cPSORTdb v 3.00 | 9.52 |
| 489765583 | LPS-assembly protein precursor | Moraxella catarrhalis strain 25240, complete genome. | OuterMembrane cPSORTdb v 3.00 | 9.99 |
| 489769126 | TonB-dependent receptor | Moraxella catarrhalis strain 25240, complete genome. | OuterMembrane cPSORTdb v 3.00 | 9.99 |
| 489769407 | phospholipase A1 | Moraxella catarrhalis strain 25240, complete genome. | OuterMembrane cPSORTdb v 3.00 | 9.93 |
| 489769419 | pilus modification protein PilQ | Moraxella catarrhalis strain 25240, complete genome. | OuterMembrane cPSORTdb v 3.00 | 9.92 |
| 490596295 | lactoferrin-binding protein B LbpB | Moraxella catarrhalis strain 25240, complete genome. | OuterMembrane cPSORTdb v 3.00 | 9.52 |
| 490597948 | peptidoglycan-binding protein LysM | Moraxella catarrhalis strain 25240, complete genome. | OuterMembrane cPSORTdb v 3.00 | 9.49 |
| 502873128 | peptidoglycan-associated lipoprotein | Moraxella catarrhalis strain 25240, complete genome. | OuterMembrane cPSORTdb v 3.00 | 9.99 |
| 740733500 | TonB-dependent receptor | Moraxella catarrhalis strain 25240, complete genome. | OuterMembrane cPSORTdb v 3.00 | 9.52 |
| 740733844 | cell envelope biogenesis protein OmpA | Moraxella catarrhalis strain 25240, complete genome. | OuterMembrane cPSORTdb v 3.00 | 9.92 |
| 740734009 | hypothetical protein | Moraxella catarrhalis strain 25240, complete genome. | OuterMembrane cPSORTdb v 3.00 | 9.52 |
| 740734079 | peptidase M23 | Moraxella catarrhalis strain 25240, complete genome. | OuterMembrane cPSORTdb v 3.00 | 9.92 |
| 740734317 | hypothetical protein | Moraxella catarrhalis strain 25240, complete genome. | OuterMembrane cPSORTdb v 3.00 | 9.49 |
| 740734435 | hemolysin activator protein (FhaC) | Moraxella catarrhalis strain 25240, complete genome. | OuterMembrane cPSORTdb v 3.00 | 9.99 |
| 740734505 | transferrin-binding protein | Moraxella catarrhalis strain 25240, complete genome. | OuterMembrane cPSORTdb v 3.00 | 9.99 |
| 740734619 | membrane protein | Moraxella catarrhalis strain 25240, complete genome. | OuterMembrane cPSORTdb v 3.00 | 9.52 |
| 740734807 | hypothetical protein | Moraxella catarrhalis strain 25240, complete genome. | OuterMembrane cPSORTdb v 3.00 | 9.49 |
| 740734813 | lactoferrin-binding protein | Moraxella catarrhalis strain 25240, complete genome. | OuterMembrane cPSORTdb v 3.00 | 9.99 |
| 740734919 | membrane protein | Moraxella catarrhalis strain 25240, complete genome. | OuterMembrane cPSORTdb v 3.00 | 9.99 |
| 740734940 | ligand-gated channel protein | Moraxella catarrhalis strain 25240, complete genome. | OuterMembrane cPSORTdb v 3.00 | 9.99 |
| 740735006 | cell surface protein | Moraxella catarrhalis strain 25240, complete genome. | OuterMembrane cPSORTdb v 3.00 | 9.99 |

Figure 20 PSORT analysis of ATCC25240 proteins localised in the outer membrane

Analysis of the ATCC25240 genome by PSORTdb: A Database of Subcellular Localizations for Bacteria. Using a combination of computationally and experimentally derived results the data base returned a list of 35 outer membrane proteins.

Table 2 List of target proteins

Combining the results of both PSORT and BOMP, and removing any duplicate proteins generated a list of 17 target proteins. Each protein was assigned an experiment ID for easier identification.

| Accession ID | Experiment ID | Protein description |
|---|---|---|
| WP_003666550 | BOMP 1 | Hypothetical protein |
| WP_038519905 | BOMP 2 | TonB-dependent receptor |
| WP_003673045 | BOMP 3 | TonB-dependent receptor |
| WP_038520226 | BOMP 4 | TonB-dependent receptor |
| WP_003659887 | BOMP 5 | MipA/OmpV family protein |
| WP_038519791 | BOMP 6 | Lactoferrin/transferrin family TonB-dependent receptor |
| WP_038520099 | BOMP 7 | Lactoferrin/transferrin family TonB-dependent receptor |
| WP_003661028 | BOMP 8 | Hypothetical protein |
| WP_003659692 | BOMP 9 | Membrane protein |
| WP_003660701 | BOMP 10 | Outer membrane protein assembly factor BamA |
| WP_038520205 | BOMP 11 | Porin family protein |
| WP_038518786 | BOMP 12 | TonB-dependent receptor |
| WP_003667703 | BOMP 13 | TonB-dependent receptor |
| WP_038519721 | BOMP 14 | ShlB/FhaC/HecB family hemolysin secretion/activation protein |
| WP_003669513 | BOMP 15 | LPS-assembly protein LptD |
| WP_038519603 | BOMP 16 | Hypothetical protein |
| WP_038519295 | BOMP 17 | BamA/TamA family outer membrane protein |

## 5.3 Analysing the target proteins experimentally

Bioinformatic analysis of the ATCC25240 genome detected 35 outer membrane proteins; of those only 17 met the experimentally indicated criteria (beta barrel structure). In order to assess the CC1 binding capabilities of the target proteins, each of the proteins were to be individually cloned into and expressed by *E. coli*. Adhesion assays utilising the transformed *E. coli* were undertaken to quantify the binding abilities of each target protein.

### 5.3.1 Selecting a plasmid

To facilitate the exploration of the target proteins CC1 binding abilities, the plasmid pOAF was utilised as a vector for *E. coli* transformations. This vector encodes the *E. coli* N-terminal signal peptide OmpA ahead of the gene of interest, which should facilitate the recombinant proteins to be expressed on the surface of *E. coli* , this allows for the CC1 binding properties of each protein to be evaluated. The presence of the lac operator with the plasmid sequence also permits for blue white screening, this process allowed for successfully transformed bacteria to be easily selected for further culture and confirmatory PCR.

### 5.3.2 Primer design

Primers which enabled ligation independent cloning for each of the target proteins were designed (table 1). In order to facilitate ligation independent cloning, each primer had a homologous overlap region with linear pOAF, at the 5' end. This sequence did

not interfere with the initial amplification of the target sequences from the ATCC25250 genomic extract.

## 5.4 Cell adhesion assays

To examine the CC1 binding capabilities of the target proteins more closely, whole-cell binding assays were performed where the target proteins were expressed individually in their native form on the surface of bacteria. In order to do this target genes were amplified from MX13L genome using the specifically designed primers. The new recombinant bacteria were used then used to conduct adhesion assays in order to determine the adherence of the bacteria to HeLa cells modified to express CC1 on their surface

### 5.4.1 Transformation of *E. coli* with target BOMPs

Using the previously designed primers a series of PCRs were performed to amplify the DNA of each of the target BOMP. As each of the primers had different melting temperatures (appendix 1) these were performed individually or in small batches using CloneAmp HiFi PCR premix. The PCR premix was specifically designed to be used in conjunction with infusion cloning due to the hi fidelity polymerase. After successful amplification the PCR products were cleaned up via the PCR clean up kit. Using the infusion cloning enzyme in conjunction with the heat shock technique the BOMPs were cloned into stellar E. coli. After a period of overnight growth on LB plates supplemented with 100 $\mu g \cdot mL^{-1}$ ampicillin, 10 $\mu g \cdot mL^{-1}$ XGal and 10 $\mu g.mL^{-1}$ IPTG several colony picks

were collected, and PCR/ gel electrophoresis performed to determine which of the colony's had been successfully transformed (figure 21). Most of the target BOMPs only produced bands of the expected size although additional faint bands could be seen in several lanes; notably BOMPs 1 and 9. An additional band could also be found in BOMP 13, this additional band was comparable in strength to the correctly amplified band. This secondary band was also present in prior amplifications of BOMP 13 and is probably caused by mis priming with a similar sequence.

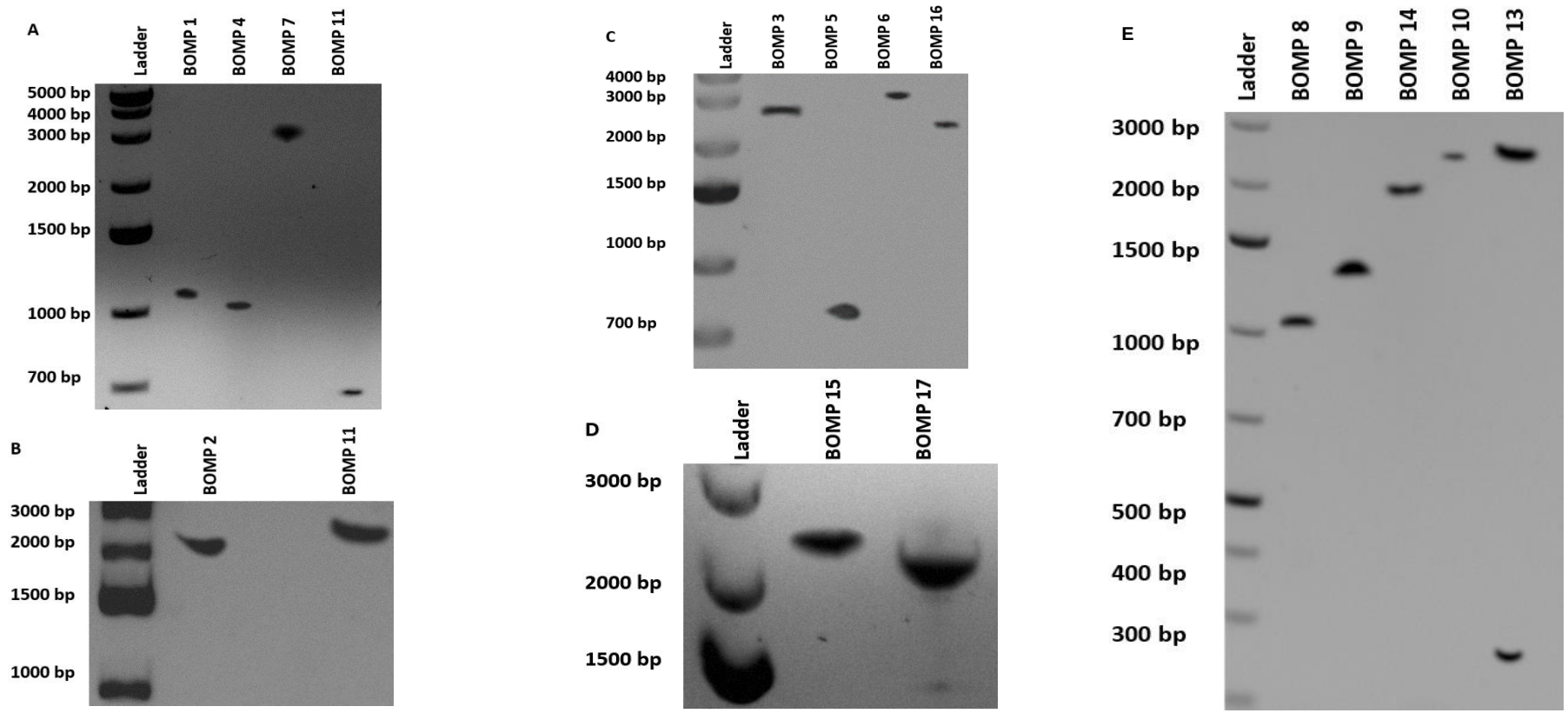



Figure 21 amplification of target BOMPs from MX13L genomic DNA.

PCR of target genes using genomic DNA from MX13L using the specific target primers (table 1). PCR product were separated using 0.8% agarose TBE gel electrophoresis. Lane 1 of each gel contained Generuler 1 kb Plus DNA ladder. Marker sizers are indicated in base pairs (bp).

Gel A: Lanes 2-5 (BOMP 1, 4, 7, & 11), produced bands at approximately 1200, 1100, 3000, and 700 bp respectively.

Gel B: Lane 2 and 4 (BOMP 2 & 12), produced bands at approximately 2300, and 2800 bp respectively.

Gel C: Lane 2-5 (BOMP 3, 5, 6, & 16), produced bands at approximately 2900, 800, 3300, and 2500 bp respectively.

Gel D: Lane 2-6 (BOMP 8, 9, 14, 10, & 13), produced bands at approximately 1300, 1400, 2000, 2500, and 2500 bp respectively. Lane 6 (BOMP 13) produced two separate products of similar intensity, the larger band at approximately 2500 bp corresponds with the expected product size. A smaller band at approximately 300 bp was also evident (this band was present in PCR obtained from MX13L genomic DNA as well as from E. coli transformed with this gene). This smaller band may be a result of mispriming with a similar sequence.

Gel E: Lane 2 and 3 (BOMP 15 & 17), both produced bands at approximately 2800 bp.

Each of the products obtained from PCR are approximate to the expected size of the genes to be amplified. Data are representative of PCR products obtained on several occasions.

### 5.4.2 Adhesion assays

In order to visually compare the binding of E.coli cells transformed to express the target proteins, adhesion assays were undertaken whereby the transformed bacteria were used to infect either HeLa CC1 expressing cells or HeLa Neo cells which did not express CC1 on their surface. There were 2 distinct sets of adhesion assays carried out, 1 set utilised RPMI media without FBS whilst the other used FBS supplemented RPMI. This was done to ensure that tertiary binding reactions which require an intermediary protein present in blood serum to interact with CC1 were also observed.

Untransformed *E. coli* cells did not appear to show any particular binding for HeLa CC1 cells compared to HeLa Neo cells, the addition of FBS to the media did not affect the background levels of adherence in any significant way either.

*E. coli* cells transformed with BOMPs 8, 14, 6 and 17 all displayed an increase in adherence to HeLa CC1 cells in both the FBS supplemented and deficient adhesion assays, compared to the background levels observed in the untransformed *E. coli* (Figure 22). BOMPs 8 and 17 also had comparatively higher levels of infection than the other two target proteins. The levels of adherence in all the aforementioned target BOMPs was also elevated when compared to that observed in the same BOMPs interactions with HeLa Neos.

Although an increase in adherence to CC1 expressing cells was evident in the adhesion assay follow up dot blots with lysates of the transformed bacteria did not show any elevated binding of CC1. The levels of adherence remained consistent with untransformed *E. coli* (figure 23), which was less than MX13L or ATCC25240.

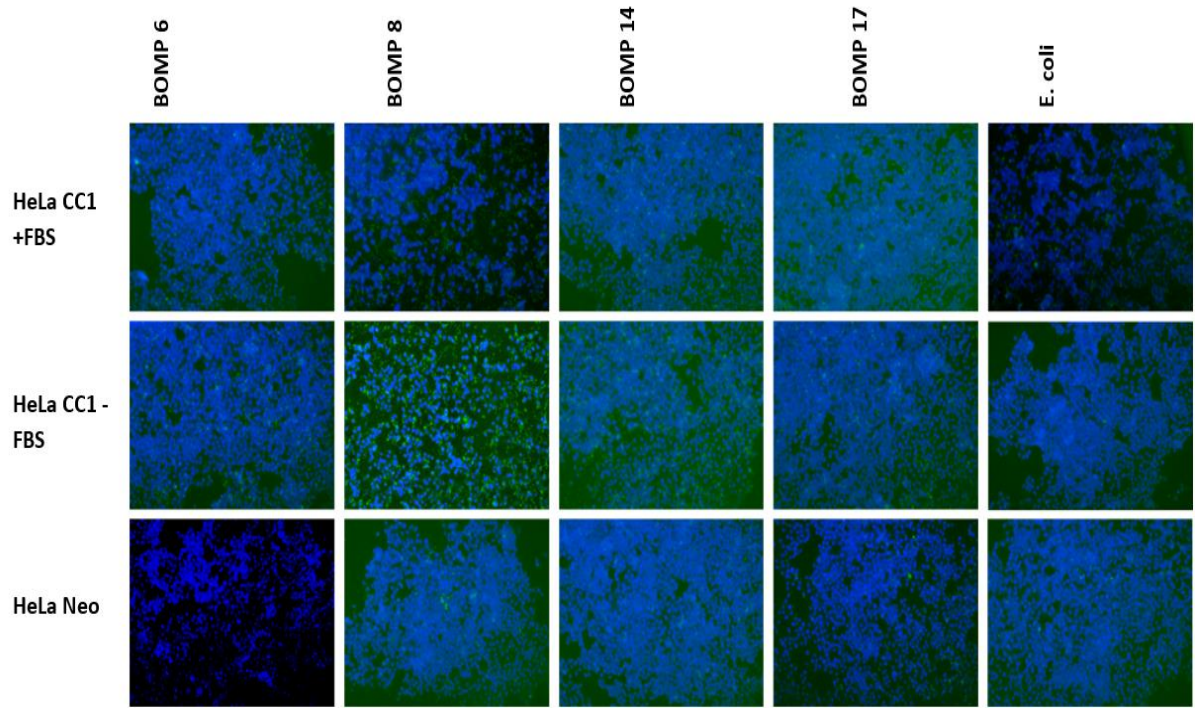



Figure 22 Adhesion assays examining the CC1 binding of *E.coli* transformed with MX13L BOMPs

An adhesion assay was performed to assess the CC1 binding capabilities of each BOMP individually under conditions which mimic a *Moraxella catarrhalis* infection (both with and without the addition of FBS). HeLa CC1 cells were infected with *E. coli* BOMP cells (MOI 100) for a period of 3 hours, before being fixed with PFA. After fixation the cells were overlaid with primary antibody Rabbit-anti-*E.coli* and secondary fluorescent antibody AlexaFluor® 488 Goat-anti-Rabbit-IgG both antibodies at a concentration of 1 $\mu g.mL^{-1}$, in order to visualise the bacterial cells. The cells were also exposed to DAPI in order to visualise the nucleus of the HeLa cells. Of the 17 BOMPs examined only four of the BOMPs displayed an increase in binding to CC1 compared to the negative control of untransformed *E. coli*.

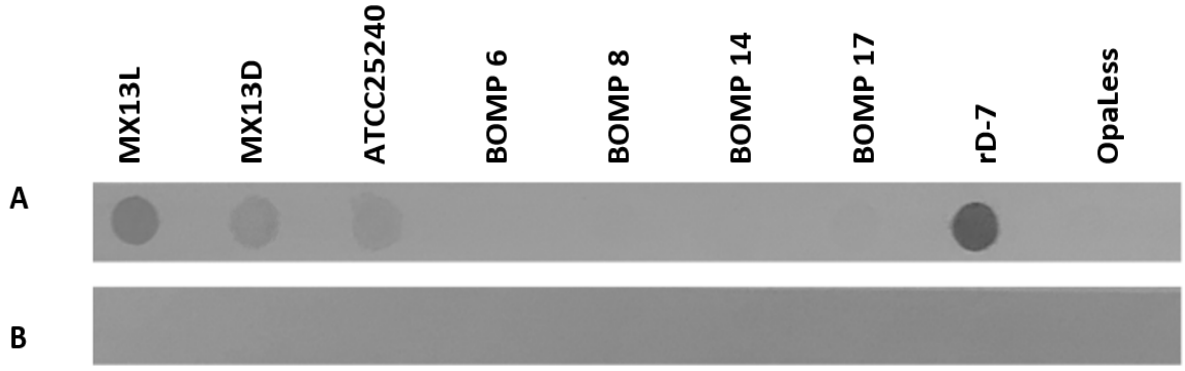



Figure 23 Adhesion assays examining the CC1 binding of BOMP expressing E. coli

A dot blot was performed in order to determine if the increase in binding to CC1 with the BOMPs 6,8, 14, and 17 observed during adhesion assays with HeLa CC1 cells, was also evident other conditions. All lysates and proteins were standardised to OD600 0.5. The lysates of selected *Moraxella catarrhalis* strains; MX13L, MX13D and ATCC25240, (dots 1-3) and E. coli expressing BOMPs 6, 8, 14, and 17 (dots 4-7) were blotted onto nitrocellulose membrane. rD-7 and OpaLess acted as positive and negative controls respectively (dots 8-9).

Membrane A was overlaid with primary antibody 1 $\mu g.mL^{-1}$ CC1, then secondary antibody 1 $\mu g.mL^{-1}$ Rabbit-anti-Human-IgG(Fc)-AP. Dots 4-7 did not appear to demonstrate any increase in binding to CC1, compared to the negative controls.

Membrane B acted as a negative control with only a secondary antibody overlay.

Data representative of products obtained on several occasions.

## 5.5 Conclusions

A daughter strain of ATCC25240, MX13L, is producing a novel CC1 binding protein which experiments indicate is beta barrel in nature. Bioinformatic analysis of the parent strain shortlisted 17 outer membrane proteins which were likely to be beta barrels, these proteins were subsequently expressed in *E. coli* and analysed for CC1 adhesion. Although four of the 17 shortlisted proteins demonstrated increased CC1 binding during adhesion assay experiments with CC1 expressing HeLa cells none of these proteins demonstrated the same increased affinity during dot blots. It was decided that it was necessary to pursue another avenue of research and a transposon library was to be undertaken.

# 6 RESULTS – TRANSPOSON LIBRARY OF *MORAXELLA CATARRHALIS* STRAIN MX13L

Due to adhesion assay experiments producing no definitive results in regard to identifying the novel CC1 binding BOMP, a transposon library was undertaken. The EZ-Tn5™ <KAN-2>Tnp Transposome™ ability to randomly 'jump' into the bacterial genome was used to create random kanamycin resistant genetic knockouts. Immunoassays were then undertaken to determine which if any of the knockouts created were in the target protein.

### 6.1.1 Colony picks

When performing a transposon library, it is important to ensure that the colonies are not too densely packed as single colony is needed per colony pick to ensure that each colony represents a single knockout gene. In order to ensure that the colonies were not too closely packed onto the surface of each plate, the 23 µL volume of transformed cells were recovered in 977 µL of SOC media, bringing the total volume up to 1000 µL. The cell media was then spread over 10 HBHI plates supplemented with 20 $\mu g.mL^{-1}$ of kanamycin.

To maximize the likelihood of identifying the knockout protein required in a transposon library, it is necessary to perform at least twice as many colony picks as there are genes in the genome of the organism being studied. As there are 1799 genes in the ATCC ATCC25240 genome (the parent strain off MX13L) and more colony picks performed increases the chance of identifying the protein of interest it was decided

that 3 times as many colony picks as there are genes in the genome would be performed. Since 10 HBHI plates were utilized for colony growth and to ensure an even spread of colony picks it was determined that the same number of colonies should be chosen from each plate. In accordance with the decision to perform three times as many colony picks as genes in the ATCC25240 genome this would have required 5.7 96-well plates per HBHI plate. To maximise the use of resources as well as make the most of the transposon library it was decided that all available wells in each 96-well plate should be filled; thus six 96-well plates were used per HBHI plate resulting in 5760 total colony picks being performed. This is greater than 3.2 times as many genes in ATCC25240.

### 6.1.2 Screening for colonies that did not bind CC1

In order to identify the target protein a series of immuno-dot blot screens were performed to determine which of the colony picks had had the target gene knocked out by the transposon. The screening process was made more complicated by the fact that a positive result for the experiment was actually the absence of binding rather than a strong binding reaction as in most experiments.

Each subsequent stage of the transposon library screening process utilised a slightly increased concentration of CC1-Fc. Screen 1 utilised a concentration of 0.2 $\mu g.mL^{-1}$ of CC1-Fc, this was in accordance with the minimum levels of CC1-Fc that the target protein can reliably bind to. This information was obtained during previous experiments assessing the binding capabilities off MX13L to CC1. Initial screens used low concentrations in order to capture any possible positive results. This was to ensure

that any isolates within this initial screen that did bind to CC1 were not eliminated due to false negatives which are more likely to occur at higher CC1 concentration. At the other end of the screening process, screen 5 utilised a much higher concentration of CC1-Fc at 1 $\mu g.mL^{-1}$, this was to eliminate false positives that had passed through previous screens. Any isolates which produced a positive result at both high and low concentrations indicated that they were true positives completely lacking any CC1-Fc binding capabilities. This variation in CC1-Fc concentrations increased the reliability of the experiment by eliminating both false negatives and false positives at either end of the screening process.

#### 6.1.2.1 Screen 1

The initial screening of the colony picks was done via hand dotting due to the number of colonies to be examined, as this process enabled more dots to fit onto a strip of nitrocellulose. In addition to the colony picks which were grown in 300 µL of HBHI broth supplemented with kanamycin series of standards were also prepared in the same manner excluding the presence of kanamycin. The standards MX13L, MX13D, rD-7 and OpaLess were used as a comparison, with rD-7 and OpaLess acting as examples of extremely strong binding and the absence of binding respectively. 2 µL of each isolate were hand dotted onto nitrocellulose paper and allowed to air dry. After blocking the non-specific binding sites with a 3% BSA solution the membranes were overlaid with 0.2 $\mu g.mL^{-1}$ of CC1-Fc. This initial screening process tested the CC1-Fc binding capabilities of all 5760 colony picks (figure 24) [appendix 2]. The majority of colony picks showed binding which was compatible either MX13L or MX13D; 59% and 28% respectively. A further 4% of the screened colony picks actually demonstrated an

increase in binding compared to their parent strain comparable to that of a functional rD-7 region. Only 489 (8%) of the isolates exhibited a lack of binding activity equivalent to that of OpaLess, which in the context of this experiment constituted a positive result. In addition to the colonies which had results comparable to that of the standards, 30 dots were also deemed unreliable. These unreliable results were identified via ponceau staining prior to the initial 3% BSA block, which showed that little or no protein was present on the membrane. Colonies which produced dots with binding comparable to OpaLess as well as unreliable dots were carried forwards into the next phase of the screening process.

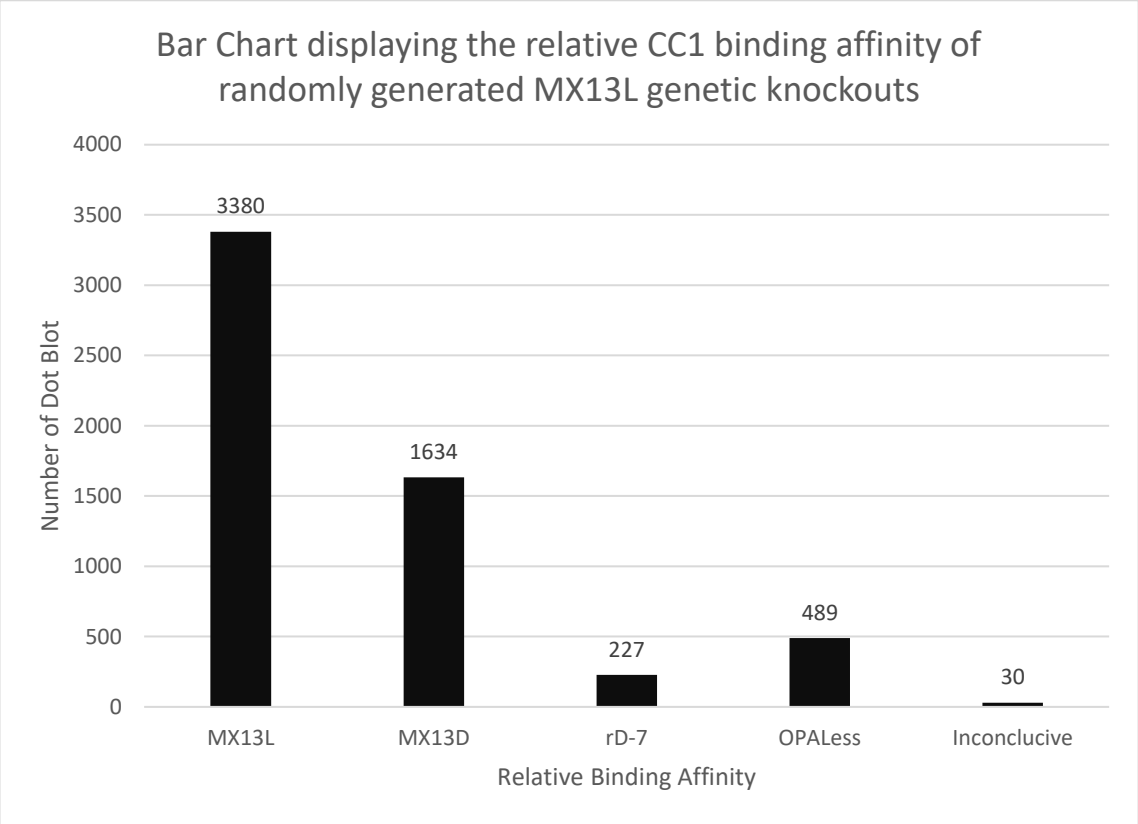


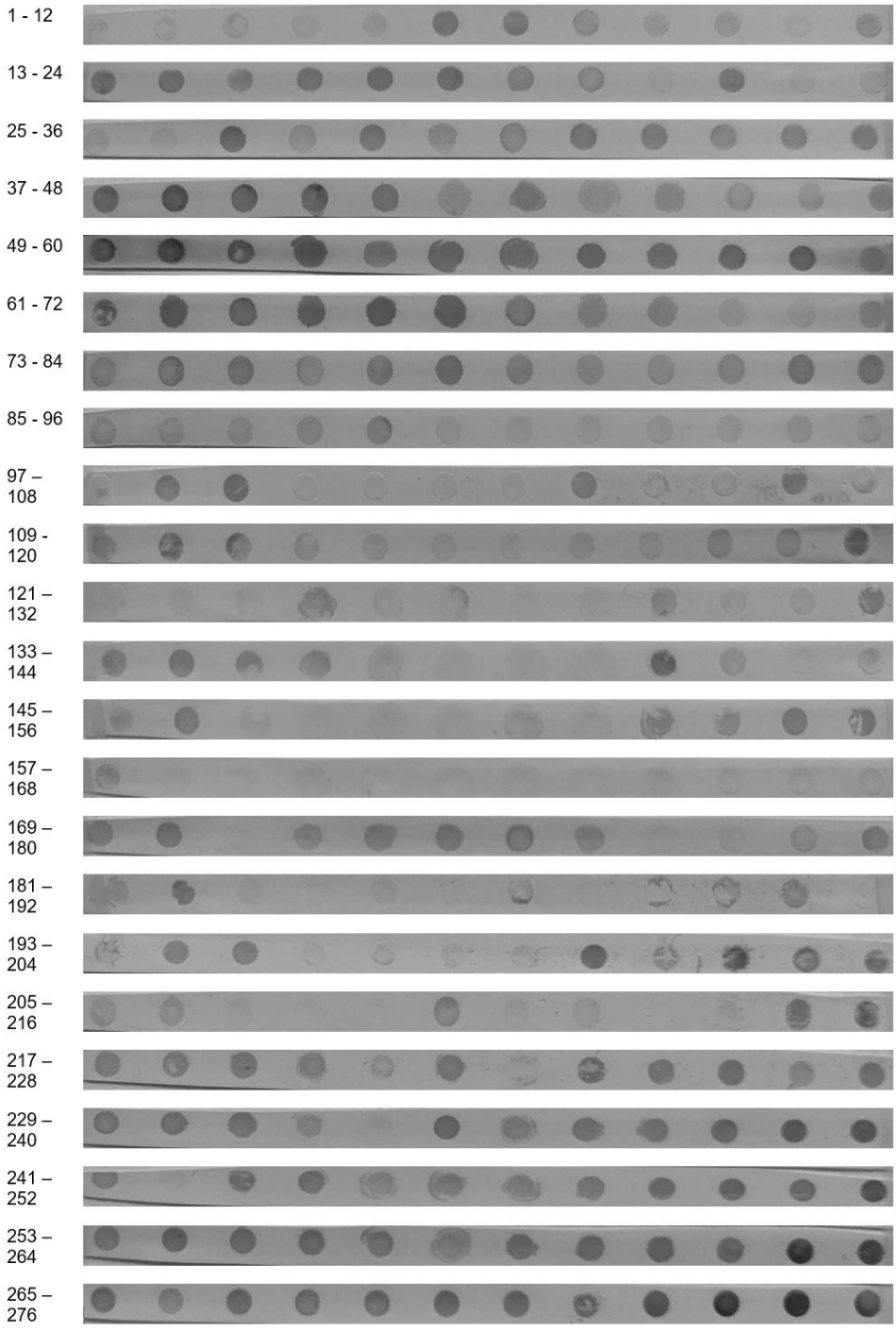

Figure 24 Graphical summary of dot blots assessing the CC1 affinity of randomly generated MX13L genetic knockouts.

A dot blot was performed to assess the CC1 binding capabilities of the MX13L knockouts created by the ez tn5 transposon. 5760 colony picks were performed, and hand dotted onto nitrocellulose membrane. The lysates of selected *Moraxella catarrhalis* strains; MX13L, MX13D, ATCC25240, MX2, as wells as rD-7 & OpaLess acted as a series of standards to compare the binding of the colony picks. The membranes were primarily overlaid with 0.2 $\mu g.mL^{-1}$ of CC1, before the secondary antibody 1 $\mu g.mL^{-1}$ Rabbit-anti-Human-IgG(Fc)-AP was applied.

The graph displays the breakdown of the total dots examined.

#### 6.1.2.2 Screen 2

A total of 486 colonies from screen 1 were carried forwards into the second stage of the screening process these colonies were grown in 5ml of kanamycin supplemented HBHI broth and following freeze-thaw lysis were standardized to $A_{280}$ OD 0.5. Using a vacuum manifold 50 microliters of each isolate were transferred to a nitrocellulose membrane and overlaid with 0.4 $\mu g.mL^{-1}$ of CC1 (figure 25). This increase in CC1 concentration indicated that 70.8% of the isolates carried forward bound to CC1. None of the isolates examined in screen 2 displayed increased binding to CC1. The isolates which did bind displayed an affinity comparable to either MX13L or MX13D. Unlike screen 1 the isolates in screen 2 were subject to freeze-thaw lysis prior to blotting, this process enabled all proteins to be released from inside the cell and maximised binding. This coupled with the increased CC1 concentration revealed that most of the isolates from screen 1 were false positives. Once again results comparable to the OpaLess strain or otherwise ambiguous results were passed onto the next stage of the screening process.


1 - 12
13 - 24
25 - 36
37 - 48
49 - 60
61 - 72
73 - 84
85 - 96
97 – 108
109 - 120
121 – 132
133 – 144
145 – 156
157 – 168
169 – 180
181 – 192
193 – 204
205 – 216
217 – 228
229 – 240
241 – 252
253 – 264
265 – 276

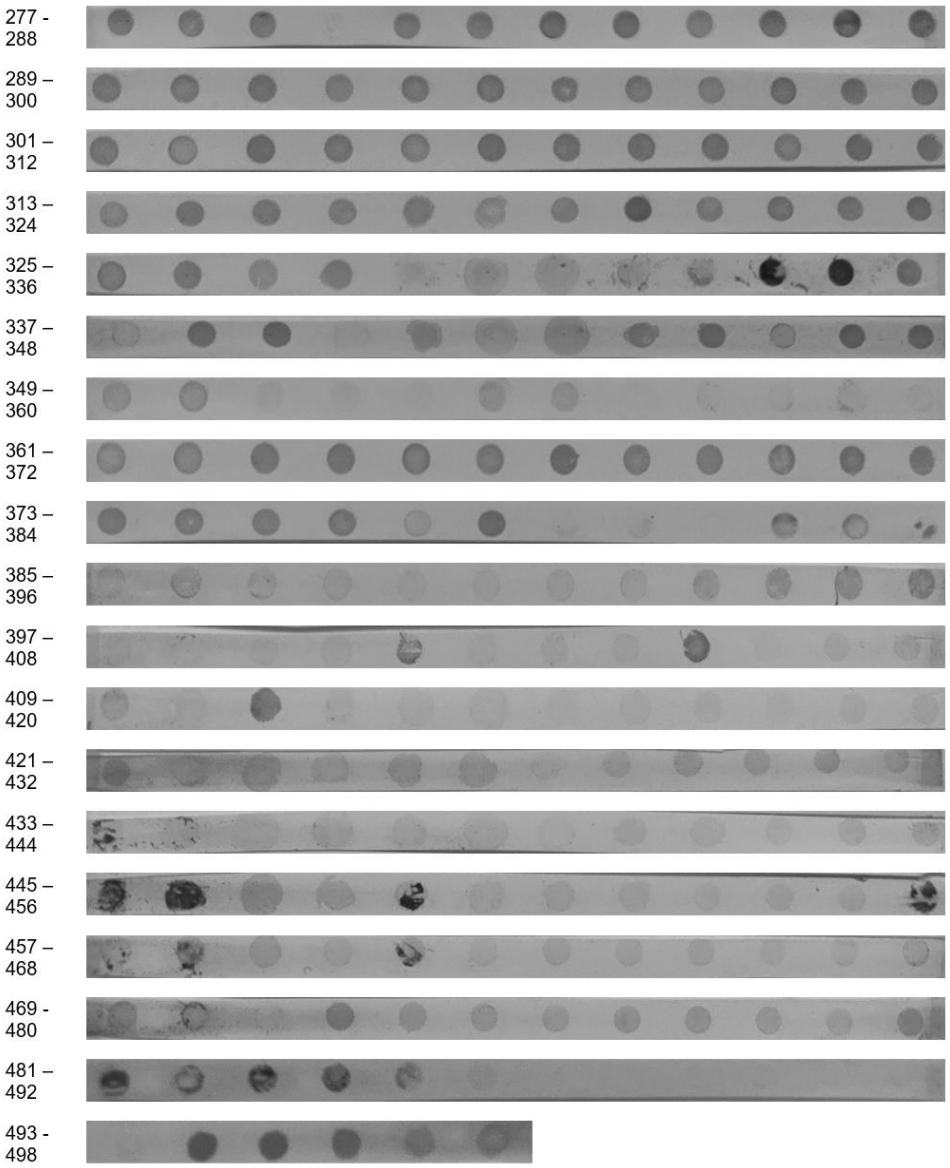



Figure 25 Screen 2 dots blots

A dot blot was performed to assess the CC1 binding capabilities of the MX13L knockouts, which showed little binding to CC1 in the previous screen. All lysates and proteins were standardised to OD600 0.5. OpaLess and rD-7 (dots 493 & 494), as well as the lysates of selected ; MX2, MX13L, MX13D & ATCC25240 (dots 495-498) acted as a series of standards to compare the binding of the colony picks. The membranes were overlaid with primary antibody 0.4 $\mu g.mL^{-1}$ of CC1, then secondary antibody 1 $\mu g.mL^{-1}$ Rabbit-anti-Human-IgG(Fc)-AP was applied.

#### 6.1.2.3 Screen 3

Screen 3 followed much the same procedure as screen 2; $A_{280}$ OD 0.5 standardised lysates of isolates that demonstrated an affinity similar to OpaLess in the previous screen were vacuum dotted onto nitrocellulose membrane and overlaid with CC1. This screen used a concentration of 0.6 $\mu g.mL^{-1}$ of CC1. 142 of the colonies from screen 2 met the requirements for examination in screen 3 (figure 26). The increased CC1 concentration revealed that of the examined isolates 82 merited further consideration whilst 9 isolates demonstrated higher levels of CC1 affinity comparable to rD-7. The rest of the isolates showed levels of binding which were similar to MX13D or MX13L.

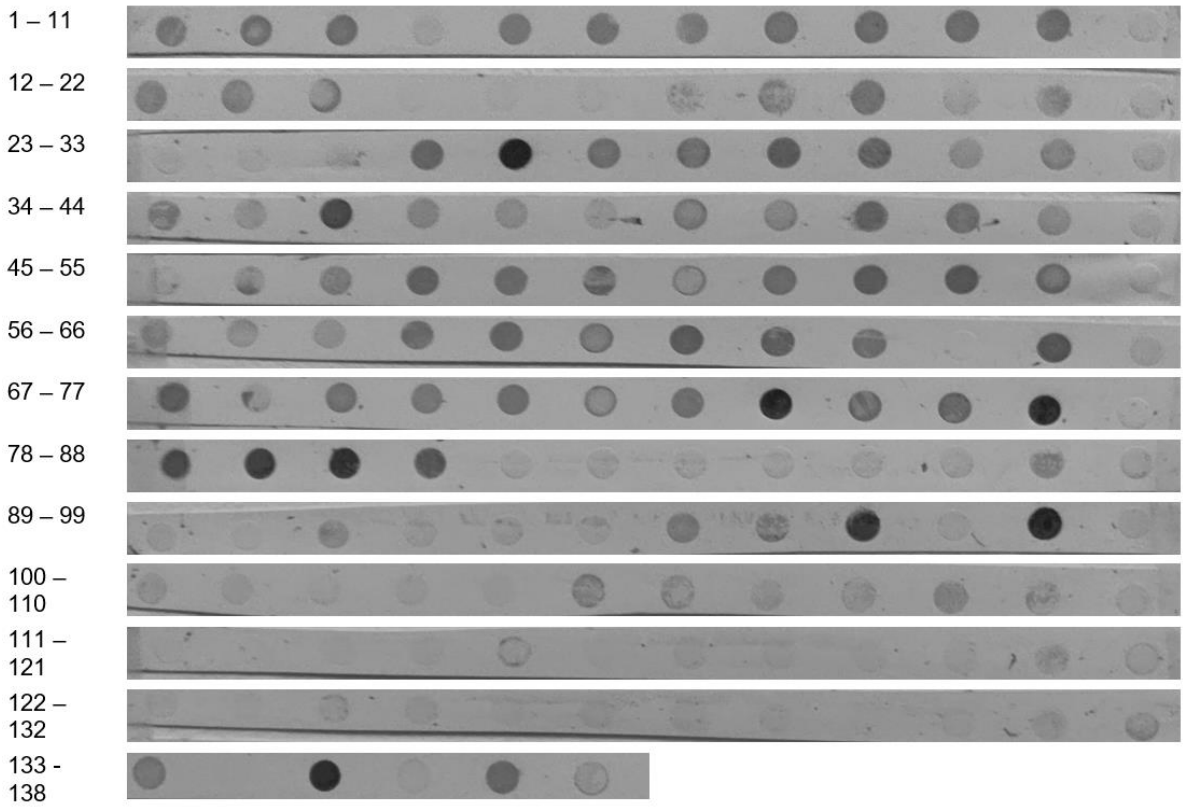



Figure 26 Screen 3 dot blot

A dot blot was performed to assess the CC1 binding capabilities of the MX13L knockouts, which showed little binding to CC1 in the previous screen. All lysates and proteins were standardised to OD600 0.5. OpaLess and rD-7, as well as the lysates of selected *Moraxella catarrhalis* strains; MX13L, OpaLess, rD-7, MX13D, Mx2, ATCC25240 (dots 133-138) acted as a series of standards to compare the binding of the colony picks. The membranes were overlaid with primary antibody 0.6 $\mu g.mL^{-1}$ of CC1, then secondary antibody 1 $\mu g.mL^{-1}$ Rabbit-anti-Human-IgG(Fc)-AP was applied.

#### 6.1.2.4 Screen 4

57.7% of the dots from the previous screen were carried forward into screen 4, as before the standardised lysates were overlaid with CC1 (figure 27). Once again, the concentration of CC1 was increased from 0.6 $\mu g.mL^{-1}$ in the previous screen to 0.8 $\mu g.mL^{-1}$ in this screen to further narrow the result base down. Of the isolates examined only 11% produced results comparable to MX13L whilst 45% were similar to MX13D. the remaining 44% of dots appeared to display no particular affinity for CC1 and thus were passed onto the next stage of the screening process. None of the dot in screen 4 displayed an affinity for CC1 that was higher than that of MX13L.

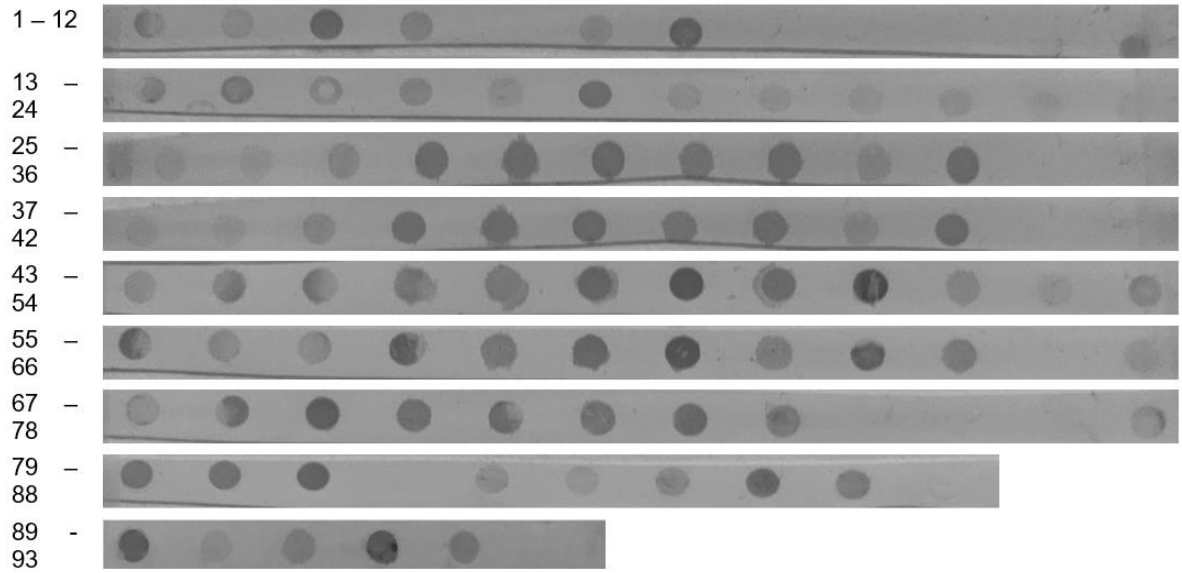



Figure 27 Screen 4 dot blot

A dot blot was performed to assess the CC1 binding capabilities of the MX13L knockouts, which showed little binding to CC1 in the previous screen. All lysates and proteins were standardised to OD600 0.5. OpaLess and rD-7, as well as the lysates of selected *Moraxella catarrhalis* strains; MX2, MX13D, ATCC25240, rD-7, Mx13L, OpaLess (dots 89-93) acted as a series of standards to compare the binding of the colony picks. The membranes were overlaid with primary antibody 0.8 $\mu g.mL^{-1}$ of CC1, then secondary antibody 1 $\mu g.mL^{-1}$ Rabbit-anti-Human-IgG(Fc)-AP was applied.

#### 6.1.2.5 Screen 5

The 5$^{th}$ stage of the screening process was another immune dot blot using 1 μg.mL$^{-1}$ of CC1, this was the maximum amount of CC1 that the novel protein would bind to whilst still displaying an affinity that was weaker than rD-7. Only one of the examined isolates displayed a stronger than MX13L affinity for CEACAM. 64% of all the colonies examined in screen 5 demonstrated binding that was comparable to MX13D, the rest appeared to have no discernible affinity for CC1 (figure 28).

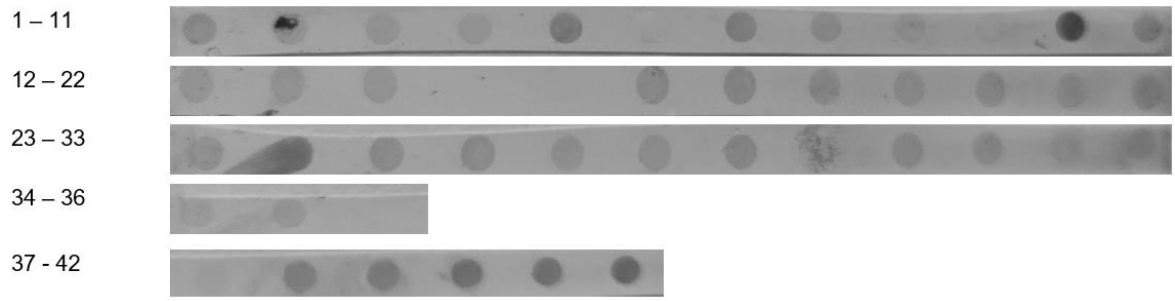



Figure 28 Screen 5 dot blot

A dot blot was performed to assess the CC1 binding capabilities of the MX13L knockouts, which showed little binding to CC1 in the previous screen. All lysates and proteins were standardised to OD600 0.5. OpaLess and rD-7, as well as the lysates of selected *Moraxella catarrhalis* strains; OpaLess, MX13D, ATCC25240, MX13L, Mx2, rD-7 (dots 37-42) acted as a series of standards to compare the binding of the colony picks. The membranes were overlaid with primary antibody 1 $\mu g.mL^{-1}$ of CC1, then secondary antibody 1 $\mu g.mL^{-1}$ Rabbit-anti-Human-IgG(Fc)-AP was applied.

### 6.1.3 Sequencing potential targets

Of the total 5760 colonies examined only 0.2% isolates appeared to have a knockout mutation in the novel CC1 binding protein. the ideal next stage of the screening process would have involved sending all 12 isolates for whole genome screening but due to budget constraints that was not possible. The last step in the transposon library screening process utilised the primers previously designed for the adhesin assay experiments. As these primers had been specifically designed to bind to the beta barrel outer membrane proteins which had been identified as the best potential targets for the novel CC1 binding protein. DNA was extracted from each of the 12 isolates which displayed no CC1 binding affinity throughout the screening process. PCR using each primer was performed, if a knockout occurred in one of the target proteins an increase of 1221bp would be seen in the agarose gel which corresponded to the size of the transposon. If no band was evident, then the transposon had possibly inserted into a region of the genome which disrupted the primer sequence.

Of the colonies examined 3.3C6, 3.3F2 and 9.6E7 (corresponding to original colony pick plate designation) displayed an increased band size in BOMP 8 from 1271 bp to 2492 bp. A further 2 isolates displayed increased bands, 4.2H5 with BOMP 14 primers and 4.3B8 with 16. The rest of the isolates did not produce any visible increased band sizes. The PCR products of the 5 isolates which resulted in increased band sizes were sent for Sanger sequencing (figure 29).

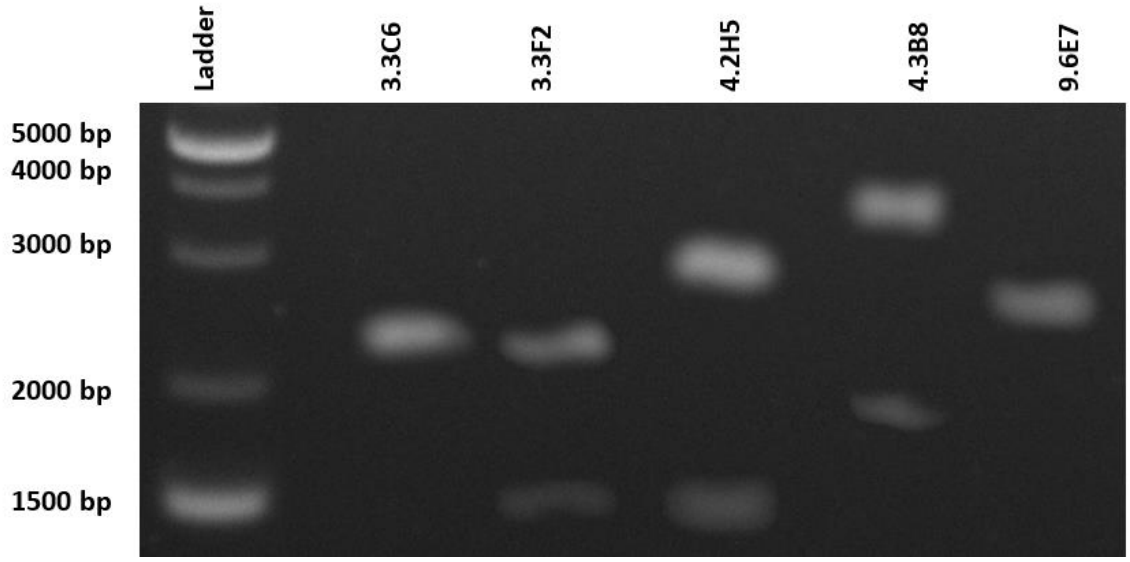



Figure 29 Amplification of target BOMPs from transposon library colonies

PCR of target genes using genomic DNA from MX13L knockout colonies, using the specific target primers (table 1). PCR product were separated using 0.8% agarose TBE gel electrophoresis. Lane 1 of each gel contained Generuler 1 kb Plus DNA ladder. Marker sizers are indicated in base pairs (bp). Lane 2, 3 & 6 (BOMP8 primers; colony 3.3C6, 3.3F2, & 9.6E7) produced bands at approximately 2500 bp, an additional faint band at 1500 bp was also observed in lane 3. Lane 4 (BOMP14 primers; colony 4.2H5) produced two bands at approximately 3000 bp and 1500 bp. Lane 5 (BOMP17 primers; colony 4.3B8) also produced two bands one at approximately 4000 bp and another at 2000 bp.

### 6.1.4 Conclusions

Unfortunately, the sequencing returned results which were inconclusive, and the sequences appeared to be corrupted. All of the sequences from the PCR amplification were shorter than the bands viewed on the agarose gel, it is possible that the samples were contaminated or degraded during transit. Due to time constrains it was not possible to repeat the PCRs and resend products for sequencing. Three out of the five colonies which increased in size with the BOMP specific primers did so with the BOMP 8 primers. This indicates that protein WP_003661028 which was designated BOMP 8 is the most likely candidate for the novel adhesin.

# 7 DISCUSSION

## 7.1 Conclusions

*Moraxella catarrhalis* was long considered a harmless coloniser of the respiratory tract but in the last few decades it has become an increasingly relevant clinical pathogen (Perez and Murphy, 2017). This bacterium is a major cause of otitis media infections, along with *Streptococcus pneumoniae* and *H. influenzae*. Unlike the conjugated duodecavalent pneumococcal vaccine (Plosker, 2013) programme for *Streptococcus pneumoniae*, *Moraxella catarrhalis* infections cannot currently be prevented with a vaccine. Recent studies examining the efficacy of the 13 valent pneumococcal vaccine have demonstrated that although the vaccine decreased the incidence of *Streptococcus pneumoniae* isolated from acute otitis media patients, the incidence of species which were not covered by the vaccine, especially *Moraxella catarrhalis* , increased (Kaur, Morris and Pichichero, 2017). *Moraxella catarrhalis* infections are on the rise with 10% of all COPD exacerbations per year implicating *Moraxella catarrhalis* species, thus increasing the bacteria's impact not only on population morbidity but also its drain on health services.

The need for more effective *Moraxella catarrhalis* treatments has never been more evident. The rapid spread of the BRO-1 and BRO-2 beta-lactam resistant phenotypes first observed in 1977 is a cause for concern. A recent study has shown that 100% of *Moraxella catarrhalis* isolates from middle ear fluid are beta-lactam resistant (Kaur, Morris and Pichichero, 2017). These beta lactamase enzymes are secreted in outer membrane vesicles which enables otherwise beta-lactam sensitive bacteria in the

vicinity to survive, complicating the treatment of polymicrobial infections greatly (Schaar et al., 2011).

In order to design a safe and effective vaccine against *Moraxella catarrhalis* more research into the bacterial mechanisms for infection must be undertaken, particularly in regard to adhesins. *Moraxella catarrhalis* main host ligand is CC1, the UspA1 and UspA2V proteins bind to CC1 using the sequence specific rD7 region. Designing a method to block CC1 *Moraxella catarrhalis* interactions would prevent *Moraxella catarrhalis* infection but in order for such treatment to be effective all CC1 interactions must be inhibited, the presence of an unknown CC1 adhesin protein would hinder the efficacy of treatments developed which only consider UspA proteins. If the novel adhesin proves to be conserved among strains as well as producing a protective antibody response it could be a potential candidate as a vaccine antigen target.

This study examined the CC1 binding capabilities of a particular strain of *Moraxella catarrhalis*, MX13L, using a combination of immune overlay assays such as dot blots and western blots. Dot blot assays identified that MX13L a strain derived from the parent strain ATCC25240, which was confirmed to be lacking a functional rD-7 region, was interacting with CC1. Western blot analysis contradicted the dot blot finding, this indicated that the interaction between MX13L and CC1 was mediated by the structural confirmation of the novel adhesin much like the OPA proteins of *Neisseria* (Martin et al., 2016).

In recent years a new field of study known as reverse vaccinology has developed, it uses bioinformatic data and computational analysis to identify potential vaccine

candidates. Bioinformatic approaches have been met with success in aiding the identification of vaccine components (Sette and Rappuoli, 2010), this process allows for the mining of genomes to identify novel antigens which are less abundant or less immunogenic during infection that are likely to be missed. This project used computational analysis in conjunction with derived experimental data enabled a short list of likely target proteins to be identified.

Using recombinant *E. coli* transformed to express outer membrane beta barrel proteins from *Moraxella catarrhalis*, adhesion assays were conducted against mammalian HeLa CC1 cells. Four proteins were identified as being probable targets: WP_038519791, WP_003661028, WP_038519721 and WP_038519295.

WP_038519791 (BOMP 6) is a lactoferrin/transferrin family TonB-dependent receptor (TonB-tbp-lbp), this family of proteins primary import iron across the outer membrane during infection (Venture Institute, 2020). This family of receptors are unique to mammalian infecting bacteria. This protein appears to be highly conserved across the *Moraxella catarrhalis* species with over 90 strains displaying greater than 98% identity between the proteins.

WP_003661028 (BOMP8) is a hypothetical protein, which although computationally predicted to exist has not yet been observed experimentally. It is a relatively small protein consisting of just 410 amino acid residues, the gene which encodes for this protein is localised within the OM channels region of the *Moraxella catarrhalis* genome. This protein is found across 60 different *Moraxella catarrhalis* strains and computational analysis marks it as part of the porin super family of proteins. Many species of bacteria use porins as adhesins, often this is a secondary function as in the case of the OmpC porin in *Shigella* (Bernardini et al., 1993). In the case of *Moraxella*

*catarrhalis* the OMP CD porin is responsible for the adhesion to A549 human lung cells (Holm et al., 2004).

WP_038519721 (BOMP14) is a ShlB/FhaC/HecB family haemolysin secretion/activation protein, it is involved in intracellular trafficking, secretion, and vesicular transport.

WP_038519295 (BOMP17) is a BamA/TamA family outer membrane protein, it is a surface antigen protein related to OMP85 from *N. meningitidis* and *N. gonorrhoeae*, and the D15 antigen from *H. influenzae*, were it is highly conserved. The D15 protein produces a protective antibody response in many animal models and shows promise as a component for a universal *H. influenzae* vaccine (Loosmore et al., 1997). This protein is also conserved amongst *Moraxella catarrhalis* strains and regardless of its involvement in CC1 binding merits further study.

Proteins of interest identified by adhesion assays were subject to further study, dot blots using the transformed *E. coli* did not confirm the adhesion assay findings. Due to the lack of definitive results with the adhesion assay experiments a transposon library was undertaken. Past studies have shown that this is an effective tool in identifying protein function. Transposon libraries have been used to identify the involvement of UspA2H in biofilm formation (Pearson and Hansen, 2007) as well as categorising the genes involved in the adherence of *Moraxella catarrhalis* to lung epithelial cells (de Vries et al., 2013).

A transposon library of the MX13L strain was produced using the ez tn5 transposon. A series of successive screens identified 12 potential isolates which did not bind to

CC1. Using the primers which were previously designed for creating the recombinant *E. coli*, 5 colonies were identified as having mutations in proteins from the target list. Three of the colonies had mutations in the same protein, WP_003661028 (BOMP 8), which had also been indicated in adhesion assays as being a likely target. Another colony from the transposon library which did not bind CC1 had a mutation in WP_038519721 (BOMP 14), this protein was also implicated in adhesion assays as being a likely target.

Sanger sequencing of the non CC1 binding transposon colonies which also displayed increased band size with the primers returned no definitive results. Due to time constraints it was not possible to send all 12 colonies from the transposon library that did not display a binding for CC1 for whole genome sequencing. In order to conclusively identify the novel CC1 adhesion all 12 of the transposon colonies which showed no binding for CC1 in screen 5 should have their genomes fully sequenced.

Although unconfirmed by sequencing, WP_003661028 (BOMP 8) remains most likely protein to be the CC1 adhesin. Of the four BOMPs expressed by *E. coli*, that showed increased binding to HeLa CC1 cells, WP_003661028 (BOMP 8) demonstrated the most adherence compared to untransformed *E. coli*. This coupled with the fact that 3 out of the 5 colonies that had an increased band size with the BOMP specific primers did so with the primers for WP_003661028 (BOMP 8), making WP_003661028 the best candidate for the novel CC1 adhesin. Further research into this novel protein is required.

## 7.2 Limitations

There are some limitations to this study; experimental evidence such as the labile nature of the protein indicated that the target may be a beta barrel outer membrane protein. All further undertakings work off of the premise that this was the case as only BOMP specific primers were designed and used.

In the case of the transposon library time constraints limited the number of colonies which could be sequenced. As such using PCR with BOMP specific primers determined that 5 out of the 12 colonies which did not bind to CC displayed an increase in product size approximate to the size of the transposon. This was an imprecise method of detection and limited the results of this study to proposing a best candidate which will need to be confirmed. Had all 12 colonies been fully sequenced this would have enabled the detection of the novel adhesion regardless of whether it was a beta barrel outer membrane protein or not.

Future experiments considering the novel CC1 binding protein of MX13L should first sequence all of the colonies which did not bind CC1, in order to conclusively identify which protein is responsible for CC1 binding.

An alternative method to identifying the target protein would have been to create KOs of the target genes in *Moraxella catarrhalis* and conduct binding assays to observe which of the newly created KOs did not bind to CC1.

As the novel protein was found in MX13L this study focused on the singular strain of *Moraxella catarrhalis* and did not seek to identify if similar binding conditions could be observed in other strains which lacked a functional UspA region. Once the protein has been fully identified via sequencing the next steps would be to use bioinformatic data

to locate other strains of *Moraxella catarrhalis* which express the gene. This would help to understand if the protein is only present in strains which do not naturally express UspA proteins or if it is ubiquitous. If the gene for the protein was present in strains which did express UspA the next stage would be to identify if the protein was always being expressed or if it was only transcribed under certain conditions.

## 7.3 Further work

Although this study identified a best candidate protein for a novel CC1 adhesin, this is only one small aspect. The completion of this project posed many more questions that should be examined in future studies.

What is the main function of the novel adhesin, is it moonlighting as a CC1 bind protein whilst having another function? Is this protein an evolutionary remnant that came before *Moraxella catarrhalis* evolved the UspA genes?

Examining other strains which also express the novel protein would further the understanding of the protein's overall function, a purely redundant protein seems unlikely so expressing the protein even in the presence of UspA would probably indicate that the protein had an additional function.

Does the presence of this protein indicate increased pathogenicity of the strain? Is it present in a specific type of *Moraxella catarrhalis* infection i.e. acute, coinfection or sepsis? Does the presence of this protein indicate increased virulence of the strain?

Once the novel protein has been confirmed, it would be possible to raise an antibody against it in an animal model. This antibody could then be used as a method to detect

the protein. Screening clinical isolates from different infection sites i.e. bacteraemia associated infection, for the presence of the novel adhesin would help determine if strains producing the protein are involved in more infections.

Does this protein represent a suitable vaccine target? Will it produce a protective antibody response?

After identification of the protein it would be beneficial to determine if it has any therapeutic relevance, outer membrane proteins which are widely distributed between bacterial strains are attractive vaccine targets. This protein should be analysed for a protective antibody response.

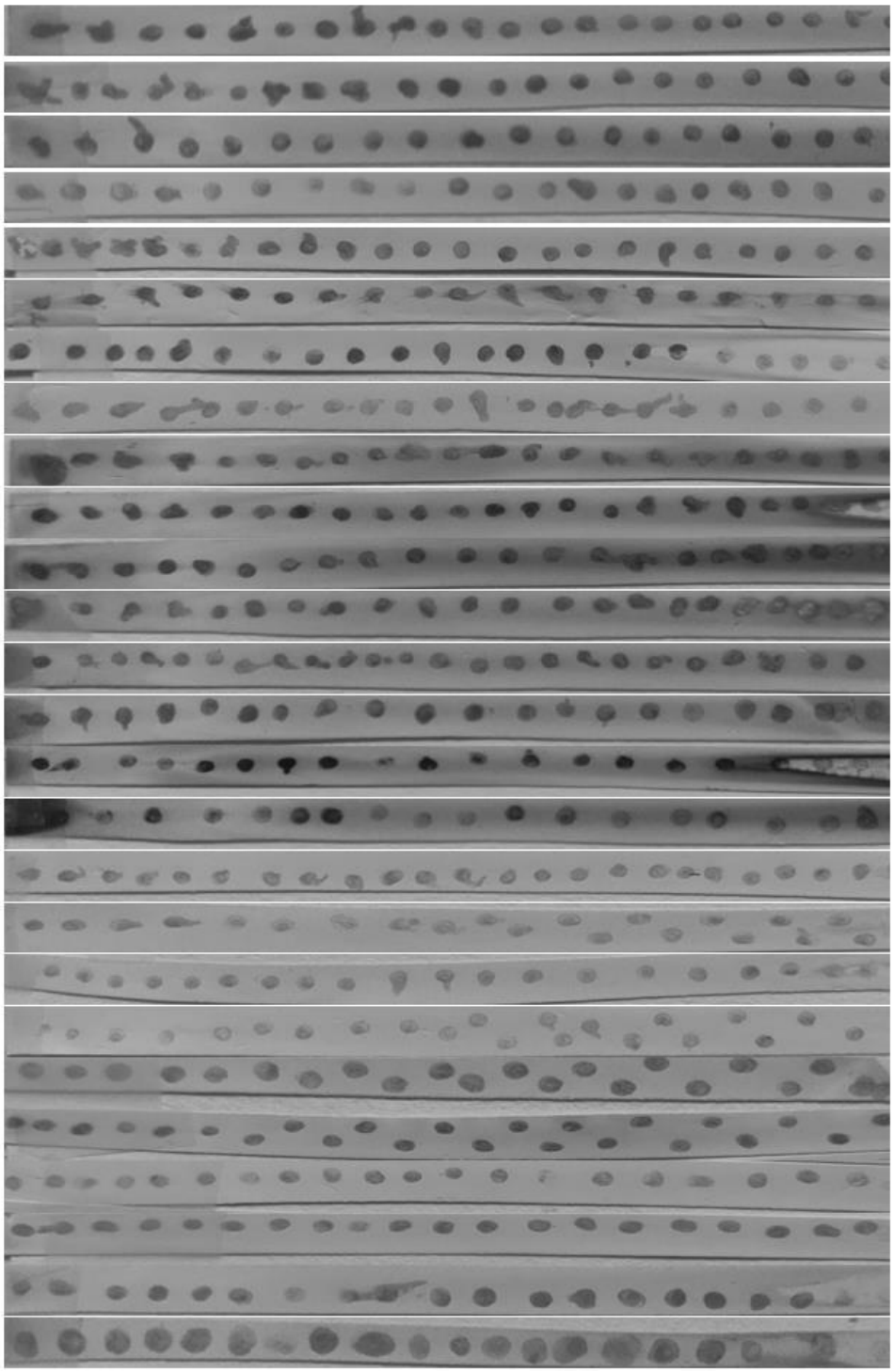
# 8 APPENDIX

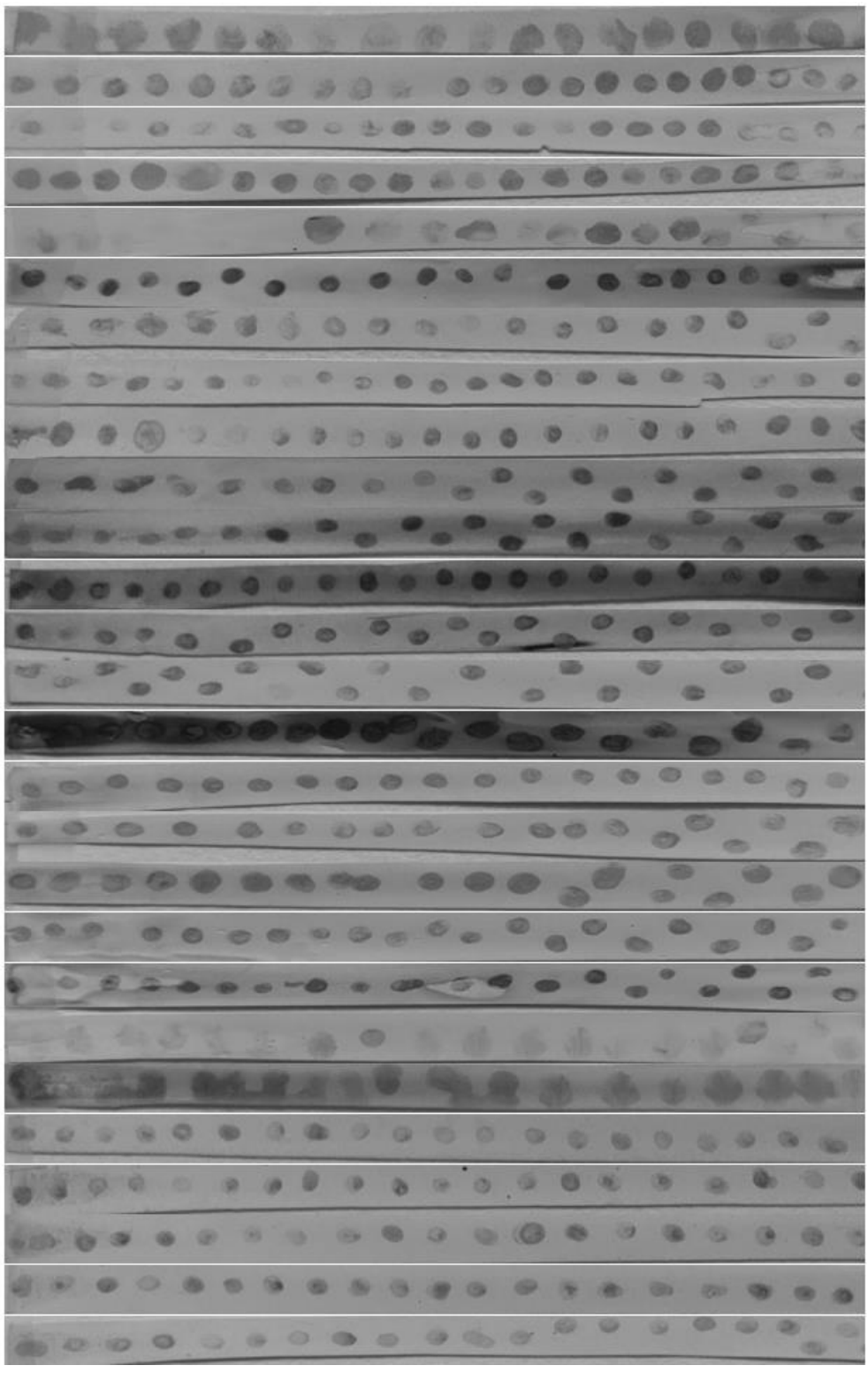
## 8.1 Appendix 1: Primers

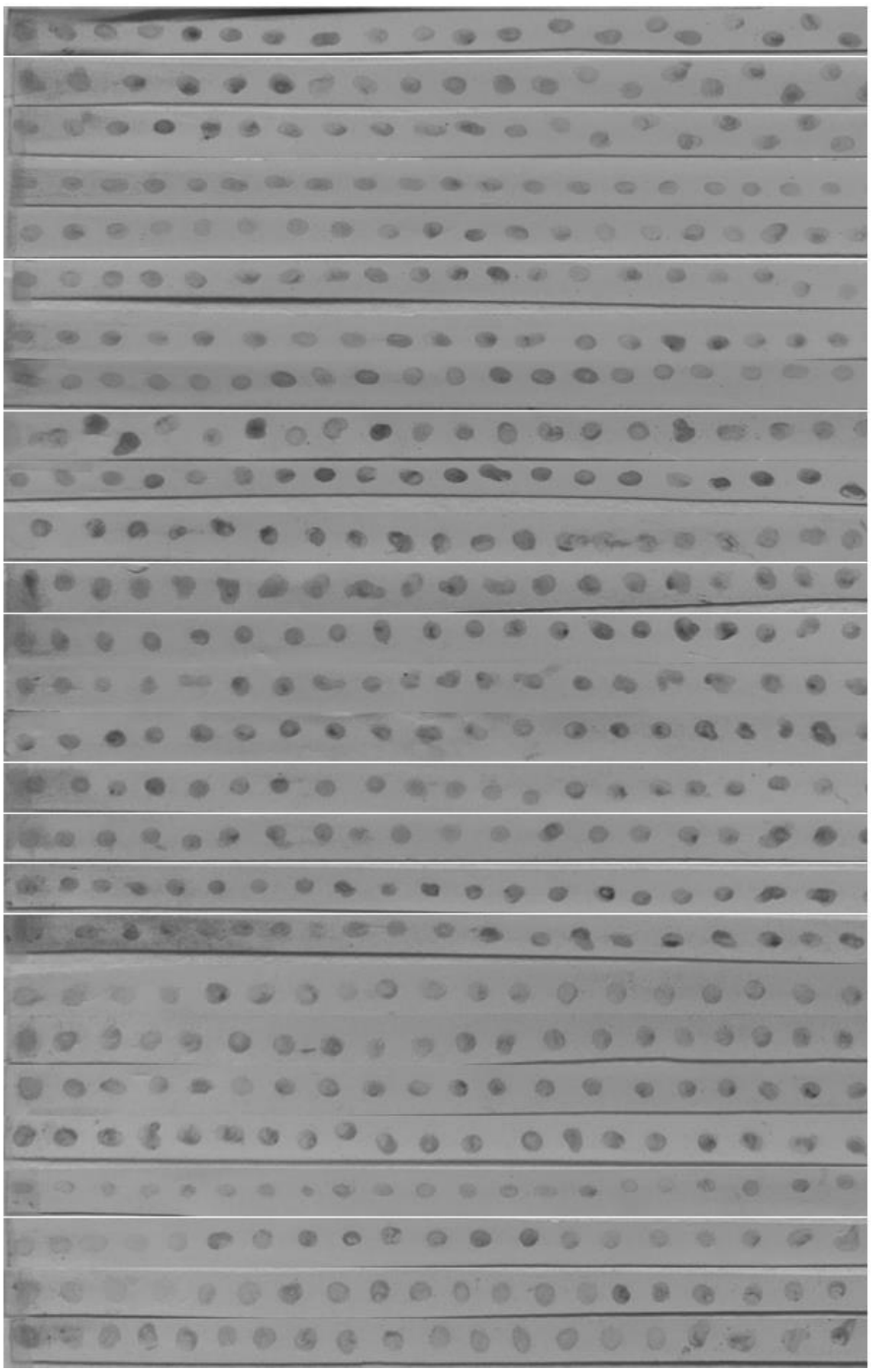
| Name | Forward Primer 5'-3' | Reverse Primer 5'-3' | Annealing Temp (°C) |
|---|---|---|---|
| **BOMP 1** | AAGTTCTGTTTCAGGGCCCGATG AAAAAAACTTCCACACAG | ATGGTCTAGAAAGCTTTATTAAAATG AAAAGCCAGTTG | 49 |
| **BOMP 2** | AAGTTCTGTTTCAGGGCCCGATG AATAAGTTTCAATTATTAC | ATGGTCTAGAAAGCTTTATTAGTAA GTGAAGTTCACGCC | 52 |
| **BOMP 3** | AAGTTCTGTTTCAGGGCCCGATG AACCAAATTTTTCATTTG | ATGGTCTAGAAAGCTTTACTACCAC TTATAAGTCATTG | 46 |
| **BOMP 4** | AAGTTCTGTTTCAGGGCCCGATG AAAAAACTTGCTCTAGC | ATGGTCTAGAAAGCTTTATTAGAATT TATATTCTAAACC | 49 |
| **BOMP 5** | AAGTTCTGTTTCAGGGCCCGATG AACATAAAAACTTTCAGC | ATGGTCTAGAAAGCTTTATTAAAAAT GATAATTTAGACC | 47 |
| **BOMP 6** | AAGTTCTGTTTCAGGGCCCGATG AATCAATCAAAACAAAAC | ATGGTCTAGAAAGCTTTATTAAAACT TCATTTCAAGTGC | 47 |
| **BOMP 7** | AAGTTCTGTTTCAGGGCCCGATG TCAAAATCTATCACAAAAAC | ATGGTCTAGAAAGCTTTATTAAAACT TCATTTCAAGACTG | 48 |
| **BOMP 8** | AAGTTCTGTTTCAGGGCCCGATG AAACCATCAATCATCAAAAAC | ATGGTCTAGAAAGCTTTATTAGAAA ATGAATGACACGCC | 51 |
| **BOMP 9** | AAGTTCTGTTTCAGGGCCCGATG AGCTTAAAATTTGGATAC | ATGGTCTAGAAAGCTTTATTATTTGG CGTGATAAGCAAG | 51 |
| **BOMP 10** | AAGTTCTGTTTCAGGGCCCGATG CGTAATTCATATTTTAAAGG | ATGGTCTAGAAAGCTTTATTAAAAG ACACTACCAATCTGG | 50 |
| **BOMP 11** | AAGTTCTGTTTCAGGGCCCGATG AAAAAATTAATTTTAGCAAC | ATGGTCTAGAAAGCTTTATTAAAAGT TATGGCGAAGAC | 48 |
| **BOMP 12** | AAGTTCTGTTTCAGGGCCCGATG ATAAAAAAACCACTTGTTTG | ATGGTCTAGAAAGCTTTATCAAAATT TAGCCGTCAAACC | 52 |

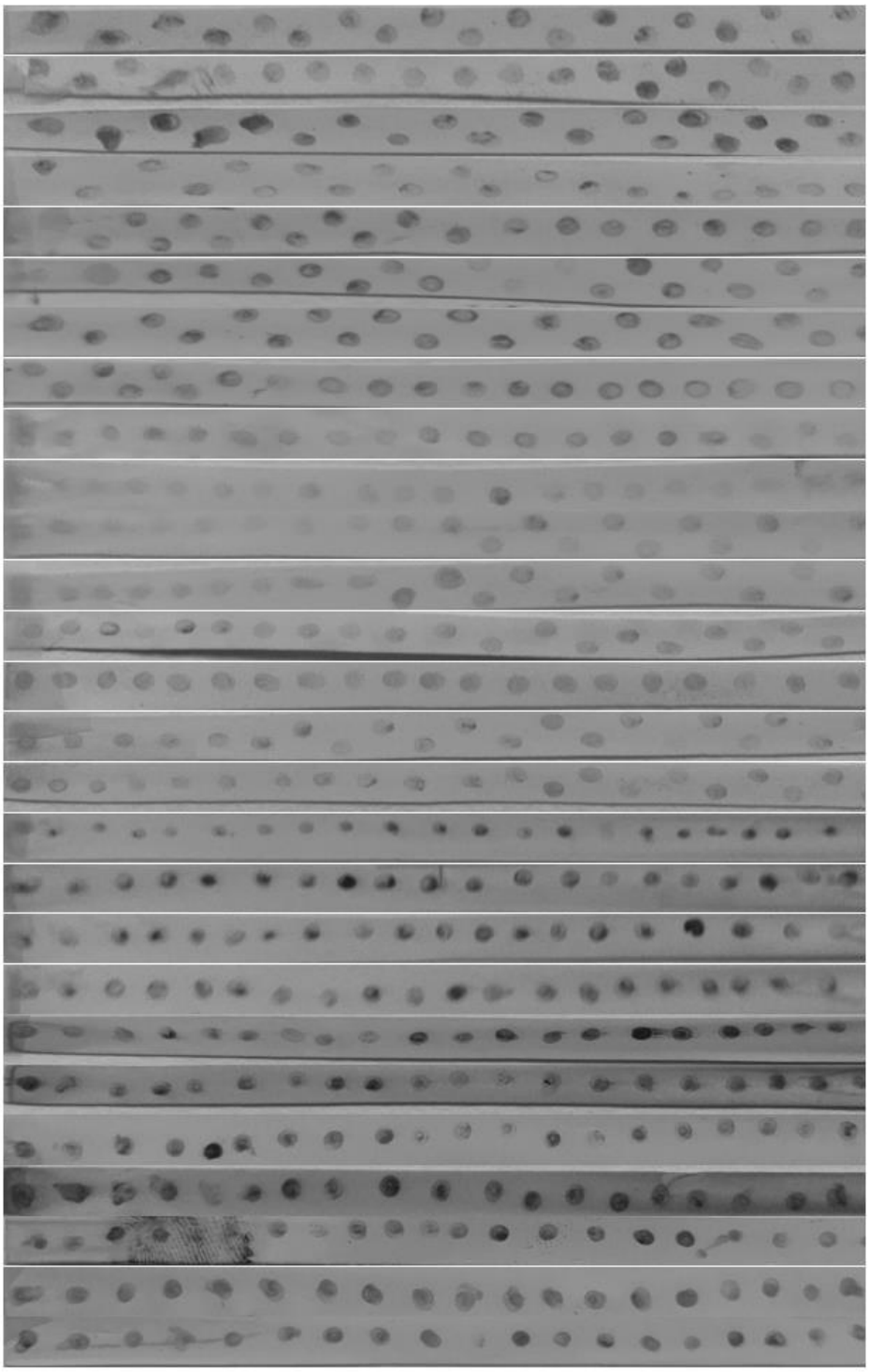
| | | | |
|---|---|---|---|
| **BOMP 13** | AAGTTCTGTTTCAGGGCCCGATG AAGGTTACCATGATAAAAAAAC | ATGGTCTAGAAAGCTTTATCAAAATT TGAAATTTGCCC | 50 |
| **BOMP 14** | AAGTTCTGTTTCAGGGCCCGATG CGTTTTATACCTTTTGC | ATGGTCTAGAAAGCTTTATTAAAACT CAATGCTGGCAC | 51 |
| **BOMP 15** | AAGTTCTGTTTCAGGGCCCGATG CACACCGCTCATCACC | ATGGTCTAGAAAGCTTTATTAGTAAT CATGTCTCCAAGC | 58 |
| **BOMP 16** | AAGTTCTGTTTCAGGGCCCGATG AGTTTTTTAAAAAAGAAAAAG | ATGGTCTAGAAAGCTTTATTAAGTG ATTAAAAGAACATC | 45 |
| **BOMP 17** | AAGTTCTGTTTCAGGGCCCGATG TCAAAGCCCGTTTTGTTTG | ATGGTCTAGAAAGCTTTATTAAAATG GTGTGCCAATAAAAAAATG | 55 |
| **UspA1** | AATGCCGCAGGTCACTTG | TTTCCAGCGGTAACTGCC | 55 |
| **UspA2** | GAAAACCATGAAACTTCTCC | ATAAGGCTGGAATAGACC | 55 |
| **CC1-Fc** | CTTGTCACGAATTCGATAATGGG GCACCTCTCAGCCCCA | GTGAGTTTTGTCAGATCTATCATTG GAGTGGTCCTG | 60 |
| **CC1** | CTTGTCACGAATTCGATAGGGCA CCTCTCAGCCCCA | GTGAGTTTTGTCAGATCTAGTGACT ATGATCGTCTTGAC | 60 |

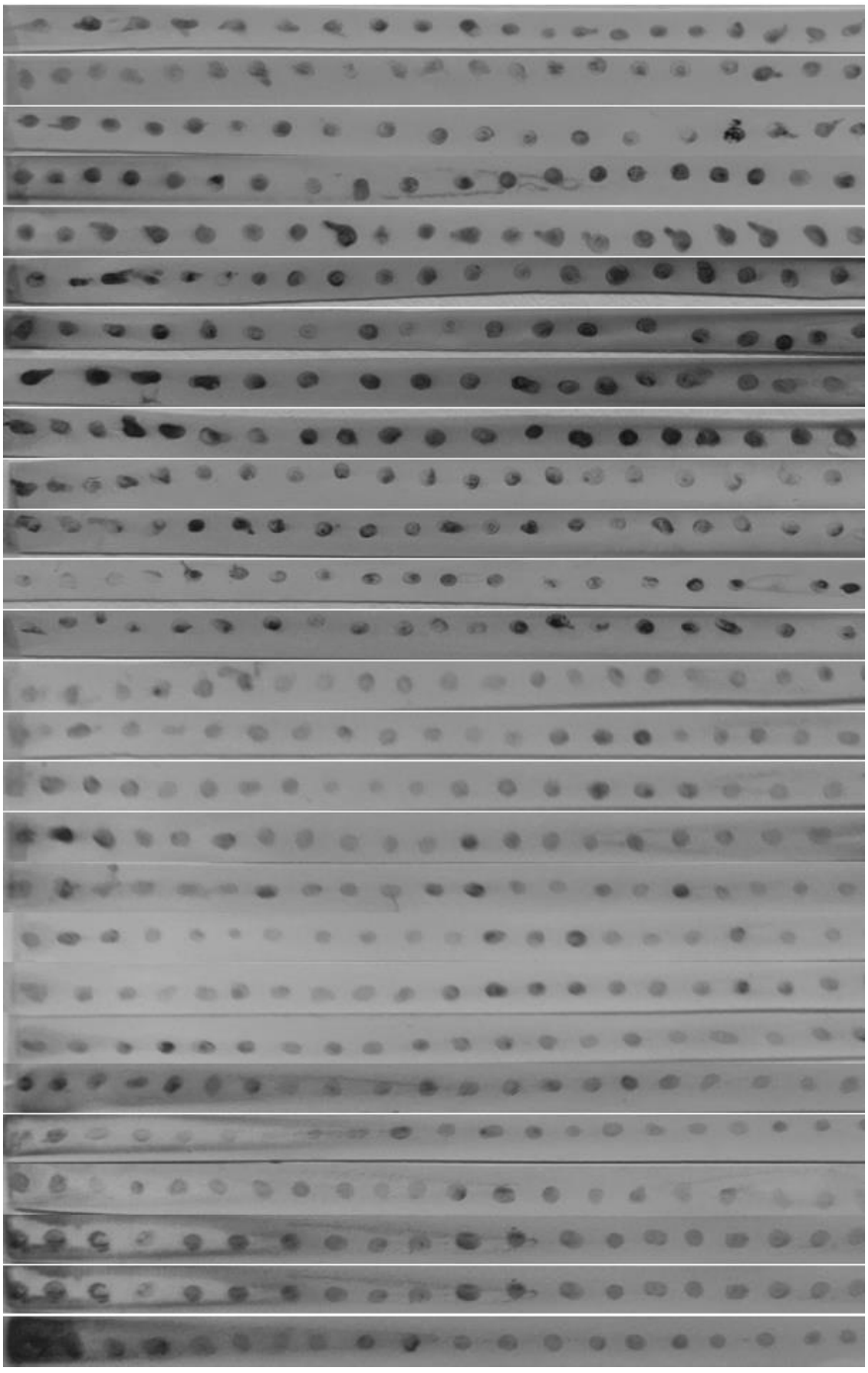
## 8.2 Appendix 2: Dot blots assessing the CC1 affinity of randomly generated *MX13L* genetic knockouts.

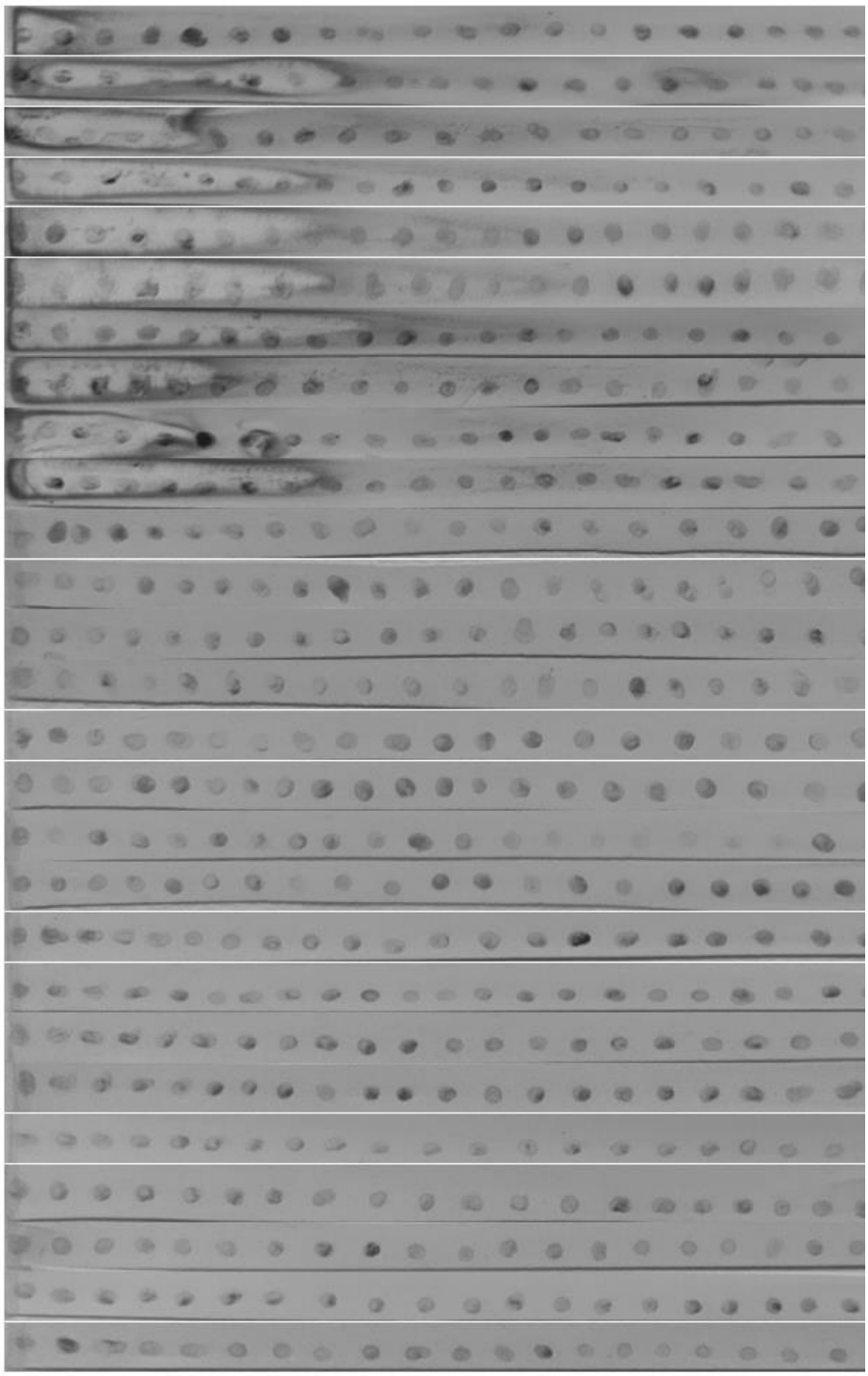


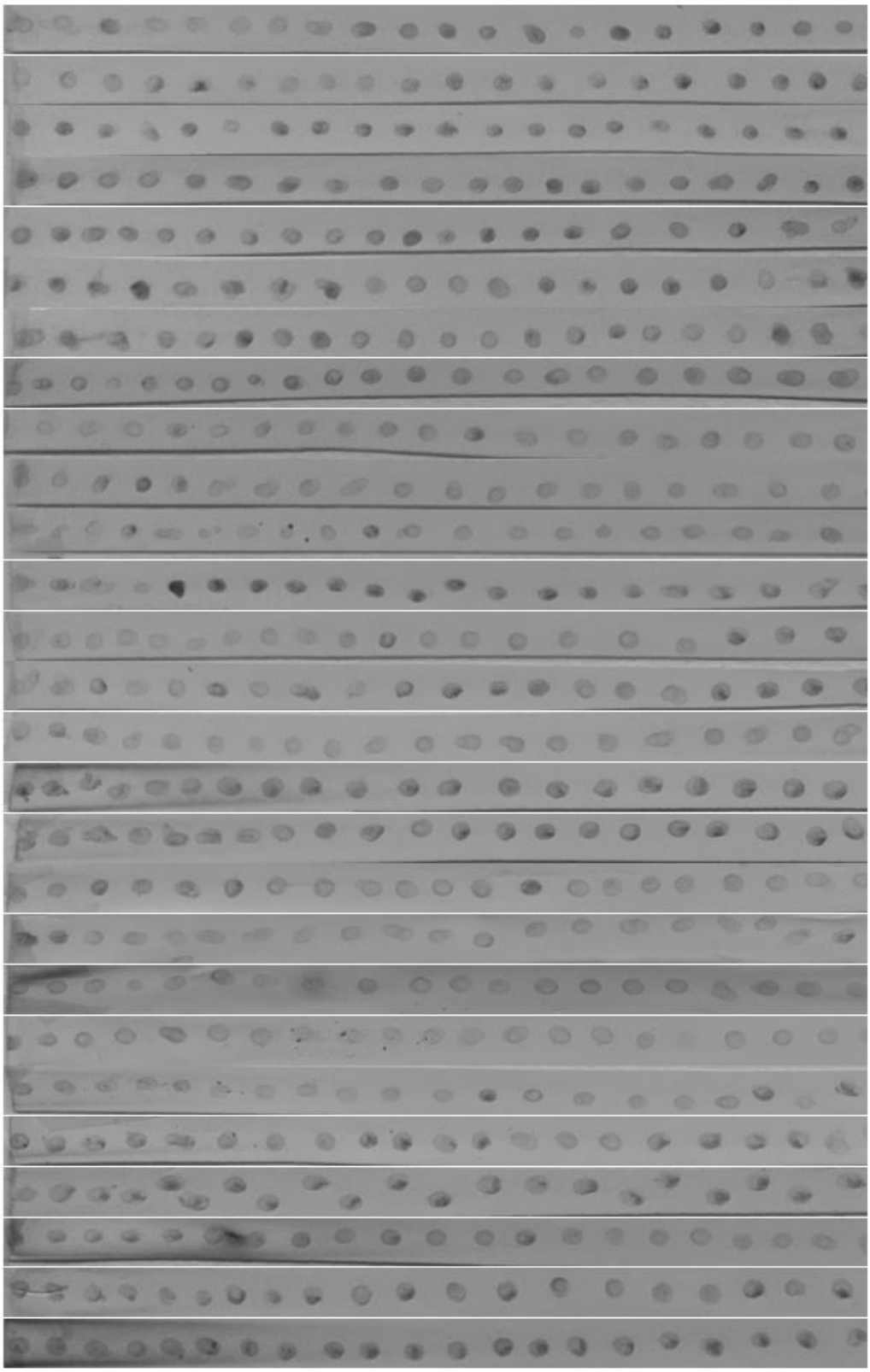


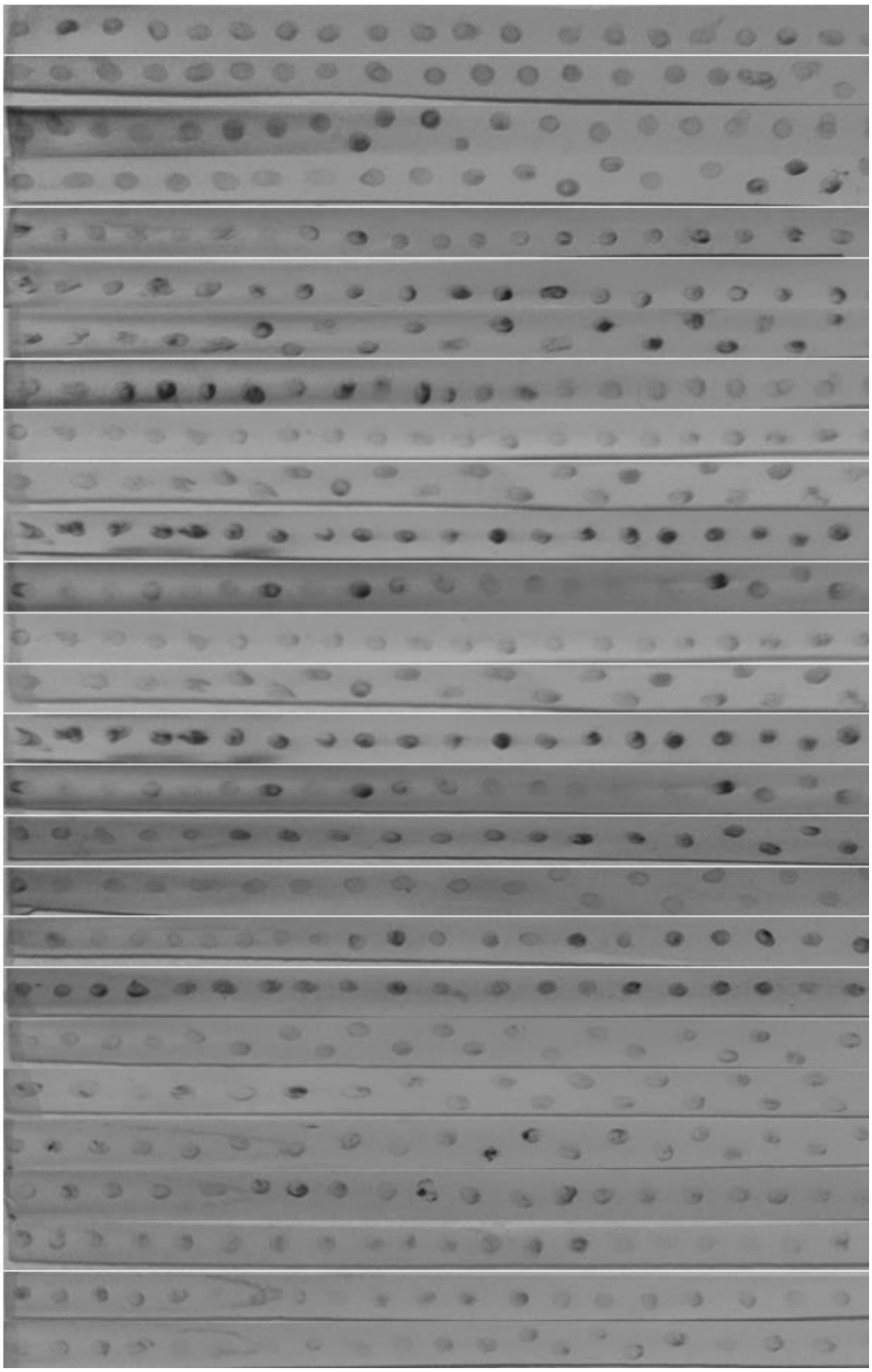


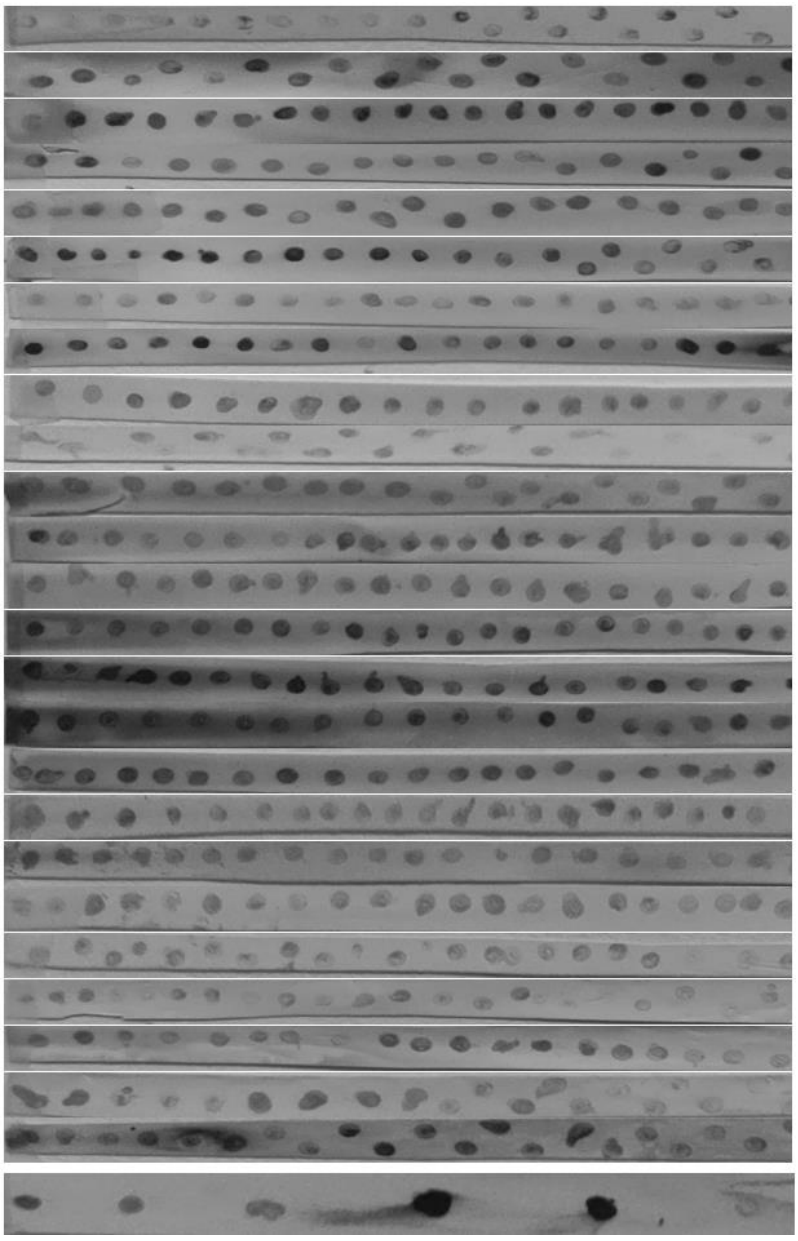












*Dot blots assessing the CC1 affinity of randomly generated* MX13L *genetic knockouts.*

A dot blot was performed to assess the CC1 binding capabilities of the MX13L knockouts created by the ez tn5 transposon. 5760 colony picks were performed, and hand dotted onto nitrocellulose membrane (dots 1-5760). The lysates of selected *Moraxella catarrhalis* strains; MX13L, MX13D, ATCC25240, MX2, as wells as rD-7 & OpaLess (dots 5761-5766) acted as a series of standards to compare the binding of the colony picks. The membranes were primarily overlaid with 0.2 $\mu g.mL^{-1}$ of CC1, before the secondary antibody 1 $\mu g.mL^{-1}$ Rabbit-anti-Human-IgG(Fc)-AP was applied. Of the total dots examined only 8% displayed binding weaker than that of MX13D and were somewhat comparable to OpaLess. Dots which were comparable to OpaLess as well as unreliable dots were passed onto the next stage of the screening process.

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